AD

Report Number 2

Pathogenesis of Cell Injury by *Rickettsia conorii*

Annual Summary Report

David H. Walker, M.D.

May 17, 1985

Supported by
U.S. Army Medical Research and Development Command
Fort Detrick, Frederick, Maryland 21701

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University of North Carolina at Chapel Hill
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This work was undertaken to determine the pathogenic mechanism by which Rickettsia conorii causes disease. R. conorii, an organism that has been neglected in spite of its widespread distribution and pathogenic qualities, was studied in human subjects, animal models, and in vitro. The purpose of the work is to elucidate the pathology of boutonneuse fever and the pathogenic mechanisms which might be blocked therapeutically or prophylactically. Human tissues were investigated by light microscopy, histochemistry, immunofluorescence, and electron microscopy. In vitro models of cell injury by R. conorii included the plaque model and cell culture release of lactate dehydrogenase.

Of biopsies of lesions compatible with tache noires from 22 patients in Sicily, 16 have been documented as BF, 1 was shown not to have BF, and 5 have incomplete data at present. Evaluation of the documented cases semiquantitatively for presence and severity of specific pathologic features yielded the following: cutaneous necrosis was present in 10 of 15 evaulatable taches noires; vasculitis was severe or moderate in all 16;

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thrombosis was severe in only 1, moderate in only 1, mild in 4, and absent in 10; dermal edema was moderate in 12, and mild in 4. The predominant leukocytes were lymphocytes and macrophages; immunofluorescent Rickettsia conorii were demonstrated in 12.

These results indicate that vascular injury by rickettsiae is the major lesion and that dermal edema is the important result. Thrombosis was generally absent or only focal and mild.

Seven consecutive Sicilian patients with boutonneuse fever who consented to liver biopsy had hepatic lesions. This suggests that R. conorii is frequently viscerotropic and in patients with particular risk factors poses a serious threat. Clinicoepidemiologic studies with European collaborators depict boutonneuse fever as geographically widely distributed and at times quite severe. The problem of developing a good animal model for boutonneuse fever has been solved only for R. conorii hepatitis in which our studies of the mouse model have progressed. We may conclude that the pathogenic mechanisms and pathophysiology of R. conorii infection are being defined at the tissue level and that the cellular level is our current goal.

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Summary

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Foreword

For the protection of human subjects, the investigator has adhered to policies of applicable Federal Law 45CFR46.

In conducting the research described in this report, the investigator adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978.

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Statement of Problem

Spotted fever group rickettsiae including Rickettsia conorii, R. sibirica, and R. akari are important potential causes of military health problems. In order to meet the challenges of these diseases to the health of groups of soldiers who enter zoonotic areas, methods of effective prevention, improved diagnosis, and optimal treatment are required. Development of an effective vaccine offers the best hope for prevention of boutonneuse fever and other spotted fever group rickettsioses. No effective vaccine exists for any of these rickettsial diseases. Because most effective vaccines for prokaryotic organisms rely upon interdiction of the specific pathogenic mechanism of the organism, e.g., diphtheria and tetanus, it is important to elucidate the pathogenic mechanism of cell injury by R. conorii. The failure of killed rickettsial and bacterial vaccines, e.g., Rocky Mountain spotted fever, typhoid fever, and cholera, may be a result of a lack of stimulation of the immune system to block crucial pathogenic steps. The goal of this research contract is to determine the pathogenic mechanism for R. conorii. Laboratory research on hypothetical rickettsial pathogenic effects must be compared with observations on the human disease in order to assure as well as possible the relevance and reality of working models of the host-parasite interaction. The problems of lack of information on the pathology of boutonneuse fever, the human ultrastructural lesions for any rickettsiosis, and the composition of the immune and inflammatory cell populations actually present in foci of rickettsial infection in humans are addressed in this research project. Diagnosis of boutonneuse fever, North Asian tick typhus, and rickettsialpox is an unsure affair with considerable room for error. Misdiagnosis and delayed diagnosis result in prolonged illness, need for more care often including nursing and hospitalization and failure to institute epidemiologic preventive illness. Yet, clinical features are variable and do not always lead to a timely correct diagnosis. There has been no rapid, acute laboratory diagnostic method. Serologic diagnosis is a retrospective tool employed during convalsescence or in the late stage of the illness. There are few facilities in the world for isolation of R. conorii, and the laboratory procedure for isolation is both cumbersome and long. A diagnostic test that can be applied during the acute stage of illness is an expected spinoff of this research project.

Background

Rickettsial diseases occur over a wide geographic distribution, are firmly entrenched ecologically, and pose an important threat to both military and public health.

Members of the genus Rickettsia are classified into three groups on the basis of shared group antigens: spotted fever group, typhus group, and scrub typhus group. All are obligate intracellular bacteria which spend at least a portion of their life in arthropod hosts such as ticks, mites, fleas, or lice. They all affect man in a similar fashion with hematogenous spread and infection of vascular endothelium producing increased vascular permeability and vasculitis in multiple organ systems. These rickettsiae include the etiologic agents of diseases that have been documented as major military health problems. Rickettsia prowazekii has affected the outcome of numerous military campaigns for centuries. R. tsutsugamushi was a severe problem in Asia and the western Pacific theaters during World War II and infected soldiers in the Viet Nam War. These rickettsiae have continued to attract research support. Although R. conorii has received far less attention, it too has been docu-

mented as an important cause of illness among troops in South Africa. R. conorii is a member of the spotted fever group of rickettsiae along with other human pathogens including R. rickettsii (Rocky Mountain spotted fever), R. akari (rickettsialpox), R. sibirica (North Asian tick typhus), and R. australis (Queensland tick typhus). Isolates of spotted fever group rickettsiae from the Mediterranean basin, where the disease is known as boutonneuse fever, East Africa (Kenya tick typhus), South Africa (South African tick typhus), and the Indian subcontinent (Indian tick typhus), were all shown to be members of the same species, R. conorii, by the mouse toxin neutralization test. Data presented by Myers and Wisseman on DNA hybridizations among the spotted fever group rickettsiae have documented close relationships among various strains of R. conorii including rickettsiae associated with the severe disease occurring in Israel and R. rickettsii. Many of these hybridizations were in the range of 90-100% homology.

Infection of man with various strains of R. conorii occurs in a widespread geographic distribution in the Old World with well-documented disease in the Mediterranean basin, Africa, and the Middle East from Israel to India. In the Mediterranean basin, the disease is endemic in Portugal, Spain, southern France, Italy, Greece, Romania, Turkey, Morocco, Algeria, Tunisia, Libya, and Egypt as well as in the margins of the Black Sea and the Caspian basin. More recently it has been reported from South Africa, Kenya, India, Pakistan, Togo, Ethiopia, Cameroun, and Israel.

In the majority of the areas where the disease is endemic, it occurs as sporadic cases during the summer months with little variation in the annual numbers of cases reported. Scafidi notes that there were 107 cases in Israel in 1974, around 30 annual cases in Tunisia from 1961-1975, and 20 annual cases in Marseille from 1925-1930. He and Bourgeade et al, however, point out that these numbers do not reflect the reality since the great majority of patients are treated at home and are not reported. This is also an explanation for the scarcity of information about the prevalence of the disease.

The low endemicity that prevails in the majority of the affected areas has changed significantly in Italy where, since 1975, there was a sharp increase in the incidence of the disease. Indeed, from an average of less than 10 cases per year up to 1972 the number of cases in Sicily increased progressively to reach 219 cases in 1979. Similar increases were observed in other regions of Italy as Liguria, Sardinia, and Lazzio; in this last mentioned region that includes the city of Rome, there were 369 cases reported in 1979 . Besides in Rome, the disease has also been reported in suburban and urban Marseille, and there are data that it is also increasing in Spain and Portugal. A large number of reports of boutonneuse fever have been published recently in Spain. Many cases are seen in southern France around Marseille every year.

The causes for such a rapid increase in the incidence of boutonneuse fever in Italy are not apparent. The Italians have suggested several possible explanations: 1) increase in the vector tick population, 2) introduction of new vectors, and 3) changes in the ecosystem. There have been some very interesting observations on the isle of Ustica where, after the recent introduction of wild rabbits, there was an explosive proliferation of Hyalomma excavatum, a tick that had rarely been found in the island previously. Gilot, et al also mention the possibility of adaptation of certain species of ticks, parasites of wild animals, to human dwellings and the potential consequences of the transmission of boutonneuse fever.

What is happening in Italy, France and Spain may occur in other regions. Weyer, reviewing the subject of rickettsioses in 1978, said, "Despite the great successes in control, none of the rickettsioses pathogenic for man have

been eradicated. Therefore, it is necessary to preserve the knowledge about these once devastating and important diseases because the present situation could change suddenly."

Indeed, recent data have demonstrated that several different species of ticks harbor R. conorii not only in the known endemic areas but also in regions where the human disease is not recognized including Pakistan, Armenia, Thailand, areas of France, Czechoslovakia, Austria, and Germany.

Boutonneuse fever is transmitted to man from ticks, most frequently by Rhipicephalus sanguineus. Infected ticks transmit the disease through their infected salivary secretions during the bite; exceptionally the agents may invade the human host from infectious tick material through abrasions in the skin or through the conjunctivae. There are references that report the disease being acquired by persons who rubbed their eyes after deticking dogs and, in fact, the principal investigator has observed just such a case. The agent appears innocuous to the tick which also serves as reservoir for R. conorii which is transmitted transovarially in ticks. Small wild mammals are the source of blood meals for immature forms of R. sanguineus. Dogs, and on occasion man, are the source of blood meals for the adult stage. The following species of ticks, besides the common vector Rhipicephalus sanguineus have been reported to harbor R. conorii: Ixodes ricinus, R. hexagonus, Dermacentor marginatus, and D. reticulatus in France; Haemaphysalis leachii, Amblyomma hebraeum, Rhipicephalus appendiculatus, R. evertsi, and Hyalomma marginatus rufipedes in South Africa; Amblyomma variegatum and Hyalomma albiparamatum in Kenya; Ixodes granulatus in Malaysia; Rhipicephalus simus, Amblyomma variegatum, A. cohaerens, and A. gemma in Ethiopia; and Rhipicephalus bursa, Hyalomma marginatum, H. lusitanicum, and Haemaphysalis punctata in Sicily. Moreover, serological tests in wild and domestic animals have shown that antibodies against R. conorii are present in several species in many regions, some of them far away from the known endemic areas. In Sicily, 20% of dogs harbor R. sanguineus and 29-71% of them have antibodies to R. conorii identified by indirect immunofluorescence assay. Serologic tests have identified antibodies against R. conorii in large numbers of healthy persons: in Africa, 13% of sera contained antibodies in an investigation in Cameroun and similar results were reported from Niger, Zaire and Central African Republic; in Greece 16% of 560 sera from healty persons were positive; data from France indicate that positive serology in healthy persons has been observed in Caen, Nantes, and Lyon. In one endemic area of Sicily 19.3% of healthy subjects had positive immunofluorescence assay for anti-R. conorii antibodies. Not all of these studies employed the same serological tests, and there is variation in specificity among different tests. Some, however, used specific immunofluorescence techniques.

All the data above presented confirm the suggestion of Weyer that the stage is set for an increase in the frequency of boutonneuse fever and that this may occur in many different areas of the world.

Recently there have been reports of cases of boutonneuse fever in German and Swiss tourists who had returned from endemic areas and even of cases in American tourists returning from Africa. Interestingly, a tick was found on one of these patients that might, if circumstances had been favorable, have become established in an American ecological niche. Cases have also been reported in persons living in Paris and other parts of Europe that are not near the Mediterranean Sea.

Human illness caused by R. conorii infection is usually an incapacitating febrile exanthem. Death has been reported more frequently in recent years, and some strains of R. conorii possess the capability of producing severe disease requiring hospitalization and critical medical and nursing care. The

disease usually resolves spontaneously in one or two weeks, this period being reduced by appropriate antibiotic therapy which may be given at home. It is necessary to emphasize that even when mild the illness is incapacitating and in a minority of cases can be severe or even fatal; moreover, in certain regions, as apparently is the case in Israel, South Africa, France, and Spain, it can assume a more severe course similar to the picture of Rocky Mountain spotted fever. Severe disease has been associated with G6PD deficiency, alcoholism, older age, and diabetes. Men are slightly more frequently affected than females, and the disease occurs at all ages being, however, uncommon in the very young and very old. Most of the patients report contact with dogs, ticks, or recent visit to endemic areas; others are farmers or hunters. The incubation period varies from 7 to 14 days, but can be as short as 4 or as long as 21 days. In the majority of the cases the patient remembers being bitten by a tick and from 33% to 92% of them have an eschar (tache noire) at the site of the tick bite. Less frequently they have acute unilateral conjunctivitis.

The disease begins with sudden increase in temperature to levels as high as 40°C; at the same time the patients complain of joint and muscle pain and violent, persistent headache that is frequently retroorbital. There is also congestion of the conjunctivae and mild lymphadenopathy. These manifestations coincide with the appearance of the eschar. Four to five days after the beginning of the fever the typical rash appears; it is first observed on the limbs but rapidly expands to trunk and face with palms and soles also being involved. In some cases even the oral mucosa presents an exanthem. In the beginning the rash appears as erythematous macules that rapidly change to a maculopapular pattern and eventually become nodular or button-like, as the name describes. The early lesions are light pink, but some of the older ones may become darker or hemorrhagic. The rash occurs in successive bouts so that lesions in different phases may be observed side by side.

Fever persists for 7-14 days, and during this period 46% of the patients develop splenomegaly, 20% hepatomegaly, and some patients, signs of pulmonary congestion. Diarrhea, constipation and vomiting may also occur. Neurological signs of meningeal irritation as nuchal rigidity or Kernig's sign as well as obtundation and even coma can be observed in a minority of the cases. These more severe manifestations occur mainly in older or debilitated persons; they are exceptional in children. Recovery is uneventful without any sequelae. Mortality is low. In a few cases, however, complications occur; they are rare and, as stated, tend to occur in older debilitated persons. Scafidi et al describe cases of hypertoxic, "dermatotifosa" and hemorrhagic disease, the last form being associated with severe gastrointestinal or genital bleeding. Fatal gastrointestinal hemorrhage with rickettsial vasculitis of the stomach has been described. Scafidi et al describe cases with atrial fibrillation, myocardial ischemia, and renal complications. A series of French publications describe "atypical rickettsiosis" with pericarditis, pleuritis, and pneumonitis. Some of the cases, however, did not present with eschars and the final diagnosis was made by positive microagglutination tests according to the method of Giroud, thus raising doubts concerning the diagnosis. In Israel, however, there have been some very interesting cases of tick-borne rickettsiosis with severe renal insufficiency requiring dialysis; in these cases, there are questions about the exact classification of the etiologic agent that did not conform exactly with the antigenic structure of R. conorii. More recently severe and fatal cases have been described in South Africa, Spain, and France.

The clinical feature that is most significant diagnostically in R. conorii infection is the tache noire which develops at the site of tickbite in

approximately 50% of cases. The tache noire, or black spot, is a zone of dermal and epidermal necrosis which may appear prior to onset of fever and rash. Conor and Burch did not describe eschars in the original report of human R. conorii infection in 1910. Tache noire is a French term and was introduced in 1925 by Pieri to refer to the tickbite site eschar in boutonneuse fever. Thereafter, the term tache noire seems to have been used continuously. Similar eschars are frequently observed in scrub typhus (R. tsutsugamushi), North Asian tick typhus (R. sibirica), rickettsialpox, (R. akari), and Queensland tick typhus (R. australis). Eschars are rarely observed in Rocky Mountain spotted fever and do not occur in typhus fever and murine typhus. Thus, eschars are seen only in rickettsioses transmitted by inoculation of infected salivary secretions by ticks and mites and are not observed in rickettsioses transmitted by scratching rickettsia-containing louse or flea feces into the skin. Patients who develop boutonneuse fever after accidental introduction of infected tick constitutents into the conjunctiva do not have eschars, but manifest conjunctivitis at the portal of entry.

Our laboratory has described the clinical features, brightfield microscopic pathology, and distribution of R. rickettsii in eschars which occurred in two fatal cases of Rocky Mountain spotted fever examined at autopsy. These eschars consisted of a 8 x 10 mm oval region of necrotic epidermis and underlying dermis. The necrotic zone was surrounded by a zone of blood vessels that were injured with extensive thrombosis and intramural and perivascular mononuclear inflammatory cells. Immunohistochemical examination revealed very large quantities of R. rickettsii in the endothelium and vascular wall of these blood vessels.

There is some degree of controversy about the role of constituents of tick salivary secretions such as enzymes associated with tickbite in the pathogenesis of the tache noire. Experimental studies suggest that the dose of inoculum of rickettsiae rather than the tickbite itself is crucial. Inoculation of a large dose of R. rickettsii, a generally nonescharogenic rickettsia, into human skin by syringe and needle produces eschars. Inoculation of R. conorii into the skin of syphilitic subjects as pyrotherapy produced taches noires proportional to the quantity of rickettsiae injected. Even nonescharogenic R. mooseri produces eschars in the skin of guinea pigs injected intradermally by syringe and needle with a large dose of rickettsiae. Not all monkeys inoculated with R. tsutsugamushi develop an eschar at the injection site; some develop only papules which do not undergo epidermal necrosis and ulceration. Rabbits inoculated intracutaneously with a high dose of R. sibirica developed an eschar; rabbits inoculated with 1% of the escharogenic dose developed only cutaneous erythema without necrosis or formation of a dark crust. Thus, the tache noire appears to be an accessible lesion that contains the pathogenic mechanisms of R. conorii and the immune and inflammatory mechanisms of the host that lead to healing.

Hypothetical rickettsial pathogenic mechanisms include both those that are host-mediated and rickettsia-mediated. Host-mediated mechanisms of injury which have been proposed include immunopathology, blood coagulation, and inflammation. Rickettsia-mediated mechanisms might include endotoxin, exotoxin, enzymes that destroy host components, metabolic competition for the host's intracellular substrates, ATP parasitism, and host cell membrane injury on rickettsial penetration into and/or release from the target cell.

Experimental evidence indicates that host-mediated pathogenic mechanisms such as immunopathology, Shwartzman phenomenon-like blood coagulation, and inflammation are not the primary mechanisms of injury in infection by R. rickettsii. Localized effects of kallikrein are probably events secondary to

the primary pathogenic mechanism(s). Occlusive vascular thrombosis is infrequent and has not been demonstrated as a primary pathogenic mechanism.

Among the hypothetical rickettsia-mediated mechanisms of injury, currently no toxin of R. rickettsii has been identified, and there is evidence against the existence of a toxin as an important pathogenic mechanism. The confusion regarding this hypothesis has originated in the so-called mouse toxin phenomenon and in erroneous analogies drawn between endotoxin and rickettsiae. Mouse toxicity depends on viable, metabolically active rickettsiae and is prevented by heating (60°C for 30 minutes), exposure to dilute formalin, rickettsial starvation, ultraviolet irradiation, specific anti-rickettsial antiserum neutralization, and a beta-lipoprotein present in some normal human sera. The pathogenesis of this phenomenon may be related to the pathophysiology of the rickettsia-host cell interaction, e.g., massive rickettsial penetration of endothelium. Rickettsiae of both the typhus and spotted fever groups have been shown to contain lipopolysaccharides. However, the endotoxin activity in bioassays including the Shwartzman phenomenon and Limulus assay was considerably less than that of potent bacterial endotoxins. Moreover, study of the adrenal in fatal RMSF has not demonstrated the pathologic lesions expected of endotoxin-mediated pathogenesis. Further evidence against the hypothesis of rickettsial toxin has been demonstrated in the plaque model. Thus, the evidence for a rickettsial toxin of pathogenic importance is quite meager.

The plaque model has been established as a useful tool for investigation of pathogenic mechanisms of cell injury by R. rickettsii. Inoculation of confluent monolayers of primary chick embryo cells derived from 12-day old specific pathogen-free, antibiotic-free, embryonated hen's eggs with a defined quantity of R. rickettsii results in a predictable course of infection and pathologic alterations in vitro. Each infectious unit under agarose overlay produces contiguous centrifugal spread of intracellular infection and injury to the host cell monolayer. This model produces a grossly visible plaque on day 5 after inoculation when a second overlay of agarose-containing the supravital dye neutral red is added. The plaque provides a temporal and spatial cross-section of the rickettsia-host cell interaction including rickettsial penetration, proliferation and release, and host cell cytopathologic alterations and necrosis. Morphometric analysis of the plaque and surrounding infected and uninfected cells has been performed maintaining the topographic relationships of the cells as a monolayer. The results have shown the association of intensity of infection and cytopathology at the microscopic and ultrastructural levels. There is a statistically highly significant relationship between the intensity of infection as measured by the quantity of intracellular rickettsiae and the presence of cellular injury as judged by cytopathology and necrosis. This relationship is valid independently of the apparent duration of infection. That is to say, more heavily parasitized host cells are more likely to exhibit pathologic alterations, even if they are located at the margin of the plaque, than those cells which contain fewer rickettsiae and are nearer to the center of the plaque. This study also confirms the observation of Silverman and Wisseman that the typical cytopathologic change in chick embryo cells infected with R. rickettsii is distinct dilation of the cisternae of endoplasmic reticulum. This ultrastructural finding is characteristic of the response of an injured cell to the influx of water. The utilization of the technique of maintaining the topography of the monolayer intact enabled us to determine that the uninfected cells of the monolayer even within 1 mm of the intensely infected marginal zone of the plaque were normal by ultrastructural and supravital dye staining criteria even though they were exposed to the same milieu of extracellular

nutritional factors, nonspecific toxic products of metabolism and substances released from injured cells, and senescence of cultured cells. Thus, the plaque model, which has a 0.5% agarose overlay that prevents rapid, distant spread of rickettsiae and yet allows for diffusion of macromolecules, demonstrated that cell injury was limited to the more heavily parasitized cells and that there was no toxic effect on uninfected cells, even those immediately adjacent. This is strong evidence that R. rickettsii does not elaborate an extracellular toxin which affects chick embryo cells. Further studies in our laboratory have extended this observation and conclusion to Vero cells which are of primate origin and to human umbilical vein endothelial cells.

Another strong indication that R. rickettsii does not produce an important toxin resulted from observations utilizing parabiotic chambers. Specially designed flasks contained coverslips with monolayers of cells with fluid overlay in separate chambers which were separated by an 0.22 um millipore filter. R. rickettsii was inoculated into one chamber of several flasks; other control flasks were observed without rickettsiae in either chamber. Inoculated monolayers developed cytopathic effect associated with heavy rickettsial infection. On the other hand, the cells in the opposite chamber remained viable with the same appearance as monolayers of unmanipulated parabiotic chambers. No toxic macromolecules injured the side of the chamber which was protected from rickettsial infection by the 0.22 um filter. The filter offered no barrier to the free passage of molecules between the infected and uninfected chambers. Thus, in an experimental system in which rickettsiae injured infected host cells, we demonstrated no effect of putative toxin, which would have been in equal concentration in the extracellular fluid of both chambers if it were present.

Examination of the hypothesis of competition for metabolic substrates has also failed to produce evidence to support it as a pathogenic mechanism in plaque model experiments with supplemental glutamate and glutamine. Although rickettsiae are capable of generating ATP for penetration of host cells by oxidation of glutamate, exogenous ATP from the host cell is utilized for biosynthesis of proteins and lipids by rickettsiae. This energy parasitism is mediated by an efficient rickettsial ATP/ADP transport system. No experiment has yet been designed and executed to test the hypothesis of energy parasitism as a pathogenic mechanism.

Experiments reported principally by Winkler and co-workers suggest that rickettsial penetration-associated phospholipase activity injures the host cell membrane. The work of Winkler and associates on hemolysis by viable R. prowazekii has led to an understanding of the rickettsia-host cell membrane interaction which probably forms the basis of penetration and a mechanism of cell injury. Rickettsial hemolysis may be divided into two steps, adsorption and lysis. Hemolysis is inhibited by cyanide (1 mM KCN, an inhibitor of the electron transport system), low temperature (0°C), and starvation of R. prowazekii for glutamate. Ghosts of erythrocytes exposed to Amphotericin B or digitonin, compounds which bind to the cholesterol-containing receptor sites in the erythrocytic membrane, are no longer able to adsorb rickettsiae. Adsorption and hemolysis are inhibited by adenine nucleotides, ADP, ATP, arsenite, which is a Krebs cycle inhibitor, and 2,4-dinitrophenol and m-chloro-phenylhydrazone, which are oxidative phosphorylation uncouplers. When rickettsiae are unable to generate ATP by metabolism of glutamate because of cyanide or arsenite inhibition, added ATP restores hemolytic activity of the rickettsiae. ATP, however, does not restore hemolytic activity inhibited by uncouplers. Fluoride (10 mM NaF) prevents hemolysis by inhibition of erythrocytic glycolysis without affecting adsorption or rickettsial metabolism. Recently, rickettsial

hemolysis has been shown to be associated with phospholipase A activity, which resulted in hydrolysis of fatty acids from the glycerophospholipids of the red blood cell membrane. Inhibition of either adsorption or lysis also prevented the release of free fatty acids.

Penetration by rickettsiae has many correlates with rickettsial hemolysis. Inactivation of R. tsutsugamushi by heat (56°C for 5 minutes), exposure to ultraviolet irradiation, or incubation with 0.1% formalin prevents penetration into host cells. Penetration of L cells by R. prowazekii comprises two steps, adherence and internalization, and requires active participation by both the rickettsia and the host cell. Treatment of rickettsiae with ultraviolet irradiation, 3% formaldehyde, or 1 mM KCN inhibited adherence to and internalization into L cells. The few inactivated rickettsiae found associated with L cells were mostly adherent rather than internalized. Treatment of L cells with NaF (an inhibitor of metabolism), N-ethylmaleimide, or cytochalasin B inhibited internalization of rickettsiae. Similar studies of the entry of R. prowazekii into endothelial cells support the hypothesis of induced phagocytosis. Inoculation of R. prowazekii onto L cells at large multiplicities of infection induced immediate cytotoxicity. This cytotoxic effect was associated with phospholipase A activity and hydrolysis of fatty acids from host cell phospholipids. Cytotoxicity and phospholipase were inhibited in a parallel manner by KCN, N-ethylmaleimide, NaF, and low temperature.

Further studies in our laboratory of pathogenic mechanisms in the plaque model employed chemical agents, which have a sound theoretical basis of inhibiting rickettsial penetration either at the step of adsorption of the rickettsia to the host cell (Amphotericin B and digitonin) or at the step of internalization associated with phospholipase A activity, have been demonstrated to reduce plaque formation. Amphotericin B and digitonin have been reported to inhibit the attachment of R. prowazekii to erythrocytic cell membranes by binding to a cholesterol receptor in the membrane. Amphotericin B was introduced in concentrations of 5 and 10 ug/ml to the overlay after the establishment of infected foci on day 4 after inoculation of R. rickettsii. In order to maintain active levels of this drug which has a decay of 50% per 24 hours at 37°C, Amphotericin B was replenished in sequential overlays on days 5 and 6. On day 6 Amphotericin B caused plaque reduction of 42-45% at both concentrations. More plaques appeared on day 7 with plaque reduction of 16-23%. A similar experiment with digitonin at the same concentrations caused similar plaque reduction (38-40%). Plaque reduction was not observed on day 7. When the levels of cholesterol receptor-binding drugs are maintained, plaque reduction can be demonstrated. This suggests that inhibition of rickettsial adsorption delays the cytopathic effect of R. rickettsii in primary chick embryo cells.

Phenterine is a drug which has been shown to have phospholipase A_2 inhibitory activity. A dose response study with this drug was performed in the plaque model. Plaque reduction was demonstrated at all doses of phentermine: 69% plaque reduction at 0.5 mg/ml; 54% at 0.1 mg/ml; 25% at 0.05 mg/ml; and 32% at 0.01 mg/ml. These results demonstrate that phentermine reduces the cytopathic effect of R. rickettsii and suggest that phospholipase activity may be a pathogenic mechanism for R. rickettsii. These data extend and support the observations of Winkler that phospholipase activity is associated with hemolysis and immediate cytotoxicity of a large inoculum of R. prowazekii.

Previous reports have documented that R. conorii forms distinct plaques similar to those of R. rickettsii in the plaque model. McDade et al produced distinct plaques with R. conorii in chick embryo cells with a first

overlay of medium 199 containing 5% calf serum and 0.5% agarose and a later second overlay of medium 199, no calf serum, 0.5% agarose, and 0.01% neutral red. Wike et al studied the critical variables in the plaque assay system for rickettsiae and also showed that R. conorii (Malish strain) produced distinct plaques in the standard chick embryo monolayer with nutrient overlay containing agarose. Thus, the plaque model offers an opportunity to examine quantitatively and predictably the pathogenic mechanisms of R. conorii in an in vitro system that may be manipulated experimentally to examine hypotheses such as phospholipase-mediated injury.

Because one hypothetical explanation for the apparent rarity of severe visceral involvement in BF as compared with RMSF (encephalitis, hepatitis, pneumonitis) is lower temperature sensitivity of R. conorii, we are interested in the effects of temperature on the physiology and pathogenicity of the organism. Oaks and Osterman have investigated the effects of temperature on the optimal growth of R. conorii. This species of rickettsia has an optimal range for growth in gamma-irradiated L cells of 32-38°C with inhibited proliferation at 40°C. The low rate of proliferation at 40°C might explain the minimal visceral involvement in febrile patients whose body core temperature is about 38°C and may exceed 40°C. An unanswered question is the effect of temperature on the pathogenic mechanism of R. conorii.

Approach to the Problem

Many features of boutonneuse fever have been investigated to a far less degree than typhus fever and Rocky Mountain spotted fever. In particular, pathogenic mechanisms, immune mechanisms against R. conorii, and the laboratory diagnosis of boutonneuse fever have not been investigated sufficiently. There are advantages of studying human cases, animal models, and cell culture models of R. conorii infection.

The localized lesion at the site of the tick bite, the eschar or tache noire, offers an excellent opportunity to extend our knowledge of pathogenic mechanisms, immune mechanisms, and laboratory diagnosis of BF in humans. In contrast to typhus and Rocky Mountain spotted fever in which the lesions, although numerous and widespread, are extremely focal, the tache noire is sufficiently large and contains a large contiguous network of severely injured blood vessels that will allow predictable sampling and qualitative and quantitative analysis of rickettsial infection, host cell injury, and host inflammatory and immune cellular response. Thus, although the brightfield microscopic lesions are better described in typhus fever, Rocky Mountain spotted fever and scrub typhus than in boutonneuse fever, these reports are not quantitative, often do not demonstrate rickettsiae with the efficiency and specificity of immunohistochemical techniques, and do not evaluate the ultrastructure of the human lesions. Surgically excised, well-fixed eschars should allow these studies in boutonneuse fever.

As yet no significant in vivo ultrastructural study of the human host-rickettsial relationship has been reported. There are two major reasons: 1) the infection in human skin is extremely focal, in the exact center of the maculopapular rash of RMSF and typhus and, thus, is difficult to find by electron microscopy; 2) intensely infected visceral tissues from fatal cases of RMSF and typhus are not suitable for ultrastructural investigation because of postmortem autolysis that occurs prior to performance of the necropsy. Surgical biopsy of the tache noire of BF should provide well-preserved lesions containing intense R. conorii infection for ultrastructural investigation. A report of the ultrastructural aspects of an eschar

in Rocky Mountain spotted fever described rickettsiae in the lesion. However, the published electron micrographs were of poor quality, and no rickettsiae were identifiable in them. Correspondence with the authors directly in an attempt to obtain copies of the original electron micrographs or the EM grids for examination personally has not been answered.

A sample of the tache noire is collected by sterile skin biopsy technique under local anesthesia after obtaining the patient's informed consent. The specimen is divided into three small 1 mm^3 blocks and fixed for electron microscopy by immersion in cold buffered glutaraldehyde-formaldehyde solution. The fixed specimen may be held in this solution for the period of shipping from Italy to our laboratory. On arrival at the Infectious Pathogenesis Laboratory in the Department of Pathology of the University of North Carolina, the specimen will be postfixed in 1% osmium tetroxide, dehydrated in graded alcohol concentrations, embedded in a mixture of Epon and Araldite, ultrathin sectioned on an ultramicrotome, and stained with uranyl acetate and lead citrate. Sections will be examined on a high resolution Zeiss 10 A electron microscope. Other transmission electron microscopes and a high resolution scanning electron microscope are also available within the departmental facilities should the need arise.

The remainder of the specimen is fixed in neutral buffered-4% formaldehyde for routine histology, histochemistry, and immunohistochemistry. Fixed tissue will be embedded in paraffin and a ribbon of serial sections will be cut at 4 um thickness. Adjacent sections will be mounted for staining by hematoxylin- eosin (H E) for routine evaluation of pathologic lesions, by phosphotungstic acid-hematoxylin (PTAH) for fibrin thrombi, by Voerhoff-van Gieson technique (VV) for evaluation of integrity of vascular elastic tissue, by modified Brown-Hopps (BH) technique for histochemical demonstration of rickettsiae, and by Giemsa technique and methyl green pyronin (MGP) for identification of host immune and inflammatory cells. Among these stains, PTAH and VV yield highly sensitive results, BH demonstrates intracellular rickettsiae but with less sensitivity, consistency, and specificity than immunofluorescence, and Giemsa and MGP assist in identification of eosinophils, basophils, neutrophils, activated lymphocytes, and plasma cells but leave a large portion of unidentified mononuclear lymphocytes.

Adjacent sections from the ribbon are processed for immunofluorescent demonstration of R. conorii. Sections are affixed onto clean glass slides with nonautofluorescent LePage Bond Fast Resin Glue to prevent them from being washed off the slide after digestion with trypsin. Sections affixed to slides with glue are heated in an oven at 60°C for 1 hour, deparaffinized in three changes of xylene for 10 minutes each, and rehydrated through serial changes of ethanol in concentrations of 100%, 95%, 70%, 50%, and 3[illegible] and finally in distilled water. Sections are then incubated in 0.1% trypsin with 0.1% $CaCl_2$, pH 7.8, at 37°C for 4 hours. The slides are washed thoroughly in distilled water, washed for 30 minutes in phosphate-buffered saline, and reacted with the specific immunofluorescent system for R. conorii. We have used anti-SFG rickettsial conjugate in the direct immunofluorescence system and indirect immunofluorescence with guinea pig immune anti-R. conorii serum followed by anti-guinea pig immunoglobulin conjugate to demonstrate structures which have the expected vascular location and coccobacillary morphology of rickettsiae.

An animal model of boutonneuse fever is needed for examination of immunity including prospective evaluation of new vaccines, for investigation of R. conorii pathogenic mechanisms and pharmacologic intervention with these pathogenic mechanisms in vivo, and for study of organ systemic patho-

physiology now that a picture of the visceral lesions is emerging for boutonneuse fever. The distributions of lesions and R. conorii need to be determined for various strains of R. conorii in mice, guinea pigs, and other species of mammals. Studies of experimental animals in our laboratory and others have shown some of the qualitative ultrastructural aspects of the rickettsia-host interaction. Our ultrastructural analysis of rickettsial infections demonstrated R. rickettsii in endothelium, vascular smooth muscle, and phagocytes of infected guinea pigs in three investigations: saline-hydration prolonged survival, antilymphocyte serum immunosuppression, and tetracycline-treated rickettsia clearance.

Further studies of pathogenic mechanisms of R. conorii are performed in the plaque model which we have exploited in investigations of pathogenic mechanisms of R. rickettsii. Aliquots of R. conorii stock are thawed and diluted in sucrose phosphate buffer to contain 500 plaque forming units (pfu) per ml. Confluent monolayers of Vero cells are inoculated with either 0.1 ml of diluted rickettsial stock containing 50 pfu of R. conorii or uninfected diluent. Rickettsial plaque technique is performed according to the method of Wike and Burgdorfer and Wike et al. After 30 minutes for absorption to occur and penetration to begin, the monolayer is overlaid with 0.5% and agarose in minimum essential medium with 5% fetal bovine serum and incubated at 35°C. On day 4 after inoculation, 4 ml of second overlay with 1% neutral red is added, and the flasks are allowed to incubate in the dark at 35°C. Flasks are examined daily for plaques afterwards with observations of monolayers by inverted microscope and with collection of specimens for examination by immunofluorescent and transmission electron microscopy.

The sides of the 25-sq cm Falcon flasks opposite the monolayers are removed by cutting the plastic. Agarose gel overlays are gently removed by separating the overlay from the sides of the flask with a sharp spatula edge and allowing the gel to detach under the force of gravity. Exposed monolayers are fixed in 70% ethanol for 20 minutes prior to direct immunofluorescent staining for R. conorii with a specific anti-R. conorii conjugate. After incubation of monolayers with conjugate for 30 minutes, they are washed in phosphate-buffered saline for 30 minutes, washed in distilled water, and mounted with 90% glycerol in phosphate-buffered saline (pH 9) and cover glass. Monolayers are examined on a Leitz Ortholux ultraviolet microscope equipped with the appropriate barrier, exciter, and edge filters for fluorescein isothiocyanate fluorescence microscopy.

Monolayers with overlays removed as described for immunofluorescence are fixed by covering the cells with a solution of buffered 2.5% glutaraldehyde for 1 hour. Cells are maintained on the plastic surface throughout postfixation in osmium tetroxide, dehydrated through a graded series of ethanol and hydroxypropyl methacrylate solutions, and embedded in Mollenhauer's Epon-araldite No. 2 followed by polymerization in an oven at 37°-45°C for 24 hours and then 60°C overnight. Embedded monolayers are separated from the plastic flasks. At this point, rickettsial plaques may be observed with the unaided eye as distinct clear zones surrounded by a grey-black carpet of cells. Plaques and adjacent cells are cut out and reembedded in flat molds with the monolayer perpendicular to the plane of sectioning. Ultrathin sections are cut on an LKB ultramicrotome using a diamond knife. Observation of the block during sectioning reveals the exact relationship of the section to the plaque. Sections are stained with lead citrate and uranyl acetate and examined on a Zeiss 10A transmission electron microscope.

Plaque size, time of appearance and phase contrast morphology are observed. The relationship of R. conorii to plaques is observed by immuno-

fluorescence microscopy. The cytopathology of injured cells is described including state of rough endoplasmic reticulum, mitochondria, plasma membrane, and nucleus. For each plaque zone (center, margin, and periphery), the quantity of R. conorii in cytopathologic and cytologically normal cells will be counted and subjected to statistical analysis for association or non-association of cytopathology with intensity of intracellular infection. The cytology of uninfected cells adjacent to the plaque will be examined.

Plaque model studies of penetration-associated pathogenic mechanism employ the plaque model, Amphotericin 3 (5 and 10 ug/ml), and digitonin (5 and 10 ug/ml), compounds which have been shown to bind to cholesterol-containing membrane receptors, to block attachment of R. prowazekii to erythrocyte plasma membrane and to reduce plaque formation by R. rickettsii when incorporated into the agarose-nutrient medium overlay. The second overlay contains the same concentration of the cholesterol-receptor binding drug. Plaques are enumerated at the time of appearance of distinct plaques in untreated plaque assays of the same inoculum for statistical analysis. In a second set of similar experiments Amphotericin B and digitonin are introduced only in the second overlay on day 4 after inoculation, at which time infected foci will be well established. Significant plaque reduction in this experiment indicates blocking of a pathogenic mechanism, not just abortion of initial infection.

One hypothesis which can be tested in the plaque model is that the paucity of signs and symptoms pointing to visceral involvement is due to a lower threshold of temperature sensitivity of R. conorii. The inability to produce pathogenic effects at temperatures greater than 38°C could explain the relative lack of severity of BF when compared with RMSF despite the 91-94% relatedness of the etiologic agents. The plaquing efficiency of various strains of R. conorii and R. rickettsii are compared at 32°C, 34°C, 36°C, 38°C, and 40°C. Variation in number of plaques formed, plaque size (area measured morphometrically by computer assisted image analysis), and time of onset are examined. These results reflect the effect of temperature on pathogenic effects.

The hypothesis of secretion of a potent extracellular toxin by R. conorii can be examined in an experiment utilizing parabiotic tissue culture chambers. Parabiotic chambers containing cell monolayers are separated by a filter with 0.22 m pore size. This filter prevents the passage of rickettsiae from one chamber to the adjacent chamber, but allows free passage of macromolecules such as metabolites, putative toxic products, or enzymes. In some pairs of chambers, one chamber is inoculated with 10 plaque-forming units of R. conorii. Other pairs of chambers are maintained with both chambers uninoculated as controls. On days 3, 5, and 7 postinoculation, trypan blue is added to selected pairs of chambers, and selected chambers are examined by immunofluorescence for R. conorii. The degree of cell injury in infected chambers, uninfected-rickettsial products exposed chambers, and control chambers is evaluated blindly by estimation of percentage of cells failing to exclude trypan blue. Immunofluorescence for R. conorii confirms the limitation of infection to inoculated chambers and allows estimation of the percentage of the monolayer that is infected.

Results

The study of taches noires from patients with boutonneuse fever has been performed in collaboration with physicians at the University of Palermo. This collaboration has proven successful with opening of several avenues for the continued investigation of the pathology, pathophysiology,

and clinical aspects of R. conorii infection in humans. The collaborative relationships with Drs. Mansueto, Tringali, and others is a valuable and productive resource for the study of R. conorii and boutonneuse fever. They are very interested in the scientific questions related to the tache noire, boutonneuse fever, and R. conorii. They have made great efforts to obtain clinical material, conduct complete clinical patient followup and convalescent laboratory diagnosis confirmation and to establish a rickettsiology laboratory. Ongoing and future investigations of pathogenetic mechanisms, epidemiology, pathophysiology, diagnostic methods and possibly preventive measures are mutually achievable goals. Considerable progress has been made toward these goals. The clinical and professional resources available in Sicily are valuable; they comprise interdependent personal and scientific relationships in a setting which offers excellent opportunities for completion of designed investigations. Drs. Mansueto and Tringali have a strong commitment to these studies, and they command an impressive ability to direct their staffs and follow their patients in the classic European style that brings about a thorough completed study.

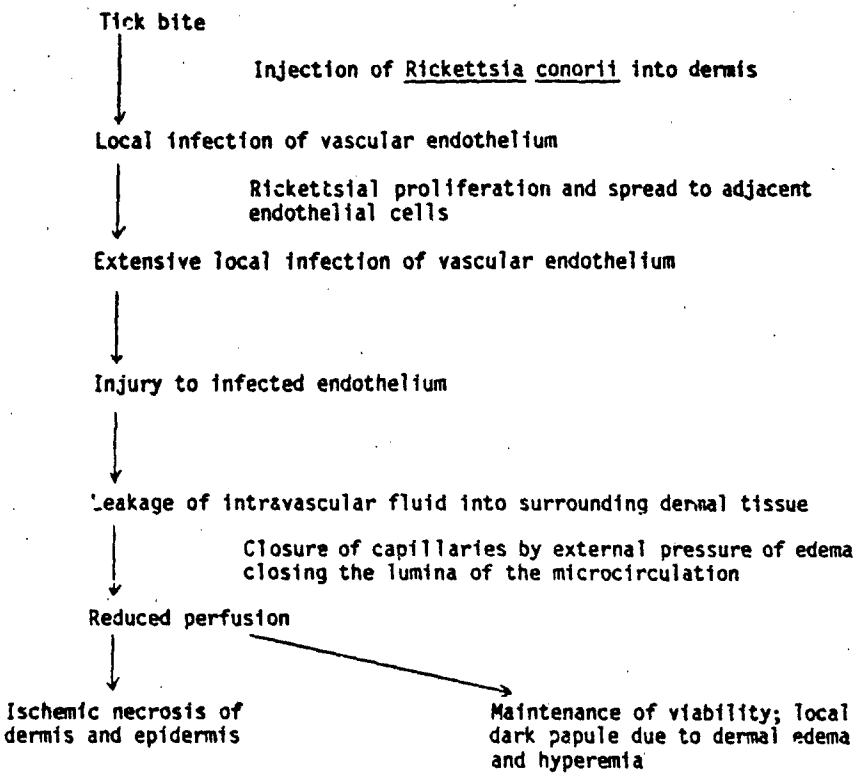
During the past year, collaboration with the clinical group of Dr. Mansueto and the rickettsiology-epidemiology group of Dr. Tringali has achieved seven principal investigations resulting in a paper presented at the American Society of Rickettsiology in Laguna Beach, California, in March, 1985; a manuscript on the frequent occurrence and clinicopathologic analysis of hepatic lesions in boutonneuse fever which has been submitted for publication; a manuscript on cold weather-season cases of boutonneuse fever with the collaboration of Dr. Raoult of Marseille which has been submitted for publication; a manuscript on familial cases of boutonneuse fever which has been submitted for publication; a manuscript on acute phase reactants in boutonneuse fever which has been submitted for publication; and a submitted manuscript on the analysis of the distribution of ticks infected with SFG rickettsiae, dogs seropositive for R. conorii antibodies, and human cases of boutonneuse fever in western Sicily.

The ASR paper on taches noires traced the history of the term and presented the results of our investigations. The term tache noire, meaning a cutaneous dark spot or eschar, was introduced in 1925 by Pieri and Boinet in France. A work published by Pieri in 1933 recounts the origin of the terminology as translated from the French publication: "In October 1925, we reported the presence of an eschar that was called the tache noire by Boinet and by ourselves and which represents, in our opinion, the portal of inoculation of the illness. The characteristic version of the tache noire comprises a black necrotic crust surrounded by an erythematous ring." The tache noire was illustrated in a series of slides of boutonneuse fever patients at the ASR meeting.

Of biopsies of lesions compatible with tache noires from 22 patients in Sicily, 16 have been documented as BF, 1 was shown not to have BF, and 5 have incomplete data at present. Evaluation of the documented cases semi-quantitatively for presence and severity of specific pathologic features (Table 1) yielded the following: cutaneous necrosis was present in 10 of 15 evaluatable taches noires; vasculitis was severe or moderate in all 16; thrombosis was severe in only 1, moderate in only 1, mild in 4, and absent in 10; dermal edema was moderate in 12, and mild in 4. The predominant leukocytes were lymphocytes and macrophages; immunofluorescent Rickettsia conorii were demonstrated in 12.

These results indicate that vascular injury by rickettsiae is the major lesion and that dermal edema is the important result. Thrombosis was generally absent or only focal and mild. Thus, the pathogenetic sequence is

likely to be:



During 1983 and 1984, seven patients suspected of having boutonneuse fever were evaluated by Dr. Staiti in Barcellona (Italy) and by Dr. Mansueto in Palermo and consented to hepatic needle biopsy. The clinical and serologic data supporting the diagnosis of Rickettsia conorii infection are presented in Table 2. Two patients had a four-fold rise in titer of antibodies to R. conorii documented by indirect immunofluorescent antibody assay (Philip et al, 1976). All seven had serum antibodies to R. conorii; five patients at a titer of 1:160 or higher. A tache noire and fever were observed in all seven patients; a rash, in five patients. Thus, the diagnosis of boutonneuse fever can be considered as confirmed in two patients and very probable in the other five. None had signs or symptoms indicative of hepatic disease.

Clinical records were reviewed for data on serum concentrations of lactate dehydrogenase, alanine aminotransferase, aspartate aminotransferase, gamma-glutamyl transpeptidase, and bilirubin.

The hepatic needle biopsies were fixed in neutral buffered 4% formaldehyde, dehydrated in a series of increasing ethanol concentrations, embedded in paraffin, sectioned at 4 um and stained with hematoxylin-eosin

as well as phosphotungstic acid-hematoxylin for fibrin, Verhoeff-Van Gieson for elastic tissue, reticulin, and Perl's Prussian blue for iron. An adjacent serial section was affixed to a microscopic slide with Elmer's glue, deparaffinized in xylene, digested with trypsin, and stained by direct immunofluorescence with a conjugate reactive with R. conorii as previously described (Walker and Cain, 1978; Montenegro et al, 1983).

The hepatic laboratory data and results of evaluation for presence of hepatic lesions and R. conorii are presented in Table 3. The moderate elevations of serum concentrations of LDH, AST, and ALT are compatible with the presence of scattered foci of hepatocellular necrosis, which involved only a small proportion of hepatocytes. The lobular location of these lesions and the absence of involvement of portal triads is reflected in that there were no striking deviations of laboratory measurements for serum bilirubin and alkaline phosphatase during the acute phase of the illness. Nevertheless, it is remarkable that all biopsies contained lesions which appeared to fit into the sequence of hepatocellular necrosis followed by focal, predominantly mononuclear leukocytic, inflammatory reaction. Yet, in no case were intact immunofluorescent SFG rickettsiae identified in the tissue. It is probable that alcoholic or other hepatic injury may have occurred as an underlying condition in some of these patients in whom fatty change was observed. This investigation confirms the reports of Guardia et al (1974) and Faure et al (1977) who have described hepatic lesions in patients with boutonneuse fever. It is our interpretation that these lesions do not fit the designation granulomatous hepatitis, but rather consist of foci of hepatocellular necrosis and predominantly mononuclear leukocytic reaction to the necrosis and probably former site of R. conorii infection. The lesion differs from a true granuloma in that it is not an avascular aggregate of activated macrophages in contrast to the granulomatous hepatitis of Q fever in which aggregates of macrophages are observed in a peculiar doughnut arrangement (Picchi et al, 1960; Pellegrin et al, 1980). C. burnetii resides within the phagolysosome of macrophages, the target cell of Q fever (Burton et al, 1971), whereas endothelial cells are the primary target of R. conorii in most organs (Walker and Gear, 1985). The target cell of R. conorii in liver awaits further study of human cases and animal models. In fatal cases of R. conorii infection in South Africa, immunofluorescent rickettsiae have been demonstrated in hepatic sinusoidal lining cells that may have been Kupffer cells or endothelial cells (Walker and Gear, 1985). Adjacent necrosis was associated with foci of rickettsial infection in those fatal cases, thus suggesting a role for rickettsiae in hepatocellular injury and the probable clearance of rickettsiae from lesions in the biopsies by effective host immune and phgocytic mechanism.

These lesions resemble multifocal hepatocellular necrosis and inflammation observed in mice infected experimentally with R. conorii (Montenegro et al, 1984). The observations that similar lesions occur in both immunocompetent and T-lymphocyte deficient mice although more rickettsiae persist in the immunodeficient mice suggests that immunopathologic mechanisms are not important in the pathogenesis of these lesions. Likewise, the fatal South African cases contained necrotic hepatic cells in foci that did not contain a cellular response. Future studies of human and experimental animal material by electron microscopy and immunohistochemistry will be important for characterization of the populations of inflammatory cells and subpopulations of T-lymphocytes as well as for identification of the hepatic target cell of R. conorii.

Finally, the most important conclusion of this study is that boutonneuse fever must not be considered as a benign disease with rare

extracutaneous involvement. The demonstration of hepatic lesions in seven consecutive patients evaluated by liver biopsy suggests that R. conorii is frequently viscerotropic and in patients with particular risk factors poses a serious threat.

Our other paper presented at the ASR meeting on the pathogenesis of SFG rickettsioses is an extension of recent reports of gastrointestinal involvement in boutonneuse fever and RMSF. Two Spanish patients with severe gastrointestinal hemorrhage have been reported by the group at the University of Salamanca. One underwent gastrectomy to control hemorrhage and subsequently died. The resected gastric specimen contained rickettsial vascular leisons as the basis for gastric hemorrhage.

Patients with RMSF frequently have nausea, vomiting, diarrhea, or abdominal pain leading to an initial misdiagnosis of gastroenteritis. Nausea, vomiting, or diarrhea early in the course were reported in 63% of a series of 131 well documented cases of RMSF. Abdominal pain was present in 34%. A lack of awareness of the gastointestinal manifestations of RMSF by the physician compounds the difficulty of dignosis during the first 2-5 days of illness prior to onset of the rash. In a study by Hattwick of 44 fatal cases of RMSF, 32% of cases, as compared with 4% of nonfatal control cases, had gastrointestinal complaints as the presenting symptoms leading to the incorrect diagnosis of gastroenteritis. In some RMSF patients, these symptoms lead to the differential dignosis of an acute surgical abdomen, and exploratory laparotomy is performed. Such manifestations of RMSF leading to laparotomy have been reported previously in at least six patients (Table 4). R. rickettsii were demonstrated by direct immunofluorescence in vascular lesions of the appendix of one patient; organism compatible with rickettsiae stained by Pinkerton's method were described in the lesions of the patient with acute hemorrhagic ulcerative ileitis.

In addition to these case reports, a recent autopsy study of the gastrointestinal tract and pancreas in RMSF was published from our laboratory. It documented rickettsial vascular lesions of vasculitis or thrombosis in stomach in 89% of the cases, in the small intestine in all cases, in the large intestine in 92%, and in the pancreas of 70%. Although it is possible for rickettsial vasculitis to result in gastrointestinal necrosis, this appears to be a rare event. We have recently investigated five patients with RMSF with interesting abdominal findings.

A 76 year old man appeared in good health until he was found drowsy and confused at his home. At a nearby hospital in western North Carolina he was noted to be febrile and extremely confused. According to his wife, he had been quite well without headache, diarrhea, nausea, vomiting, abdominal pain or rash. Clinical and laboratory evaluation during the subsequent four days failed to establish a diagnosis. There was no skin rash. He remained febrile and was treated empirically with cefoxitin, gentamicin, and erythromycin for presumed sepsis. On his fifth day of hospitalization, he complained of pain in the right side of the abdomen, and an abdominal x-ray revealed ileus and possible cholelithiasis. Because of the abdominal pain and abnormal radiograph, acute cholecystitis was suspected and exploratory laparotomy was performed revealing cholelithiasis, a non-obstructive stone in the common bile duct, and hepatomegaly, but no evidence of cholecystitis, abdominal or retroperitoneal abscess, or appendicitis. The stone was removed. Cholecystectomy and appendectomy were performed. His postoperative course included persistent fever, seizures, coma, hypotension, acidosis, thrombocytopenia, and oliguria progressing to anuria. Postoperative laboratory data included elevated concentrations of hepatic enzymes, and serum bilirubin. Metronidazole, cefoperazone, and platelet transfusions

were added to his treatment regimen. After transfer to another hospital for hemodialysis, physical examination revealed a suggestion of an erythematous macular rash with scattered petechiae, purpurae, and peripheral pitting edema. A skin biopsy contained immunofluorescent Rickwettsia rickettsii. The was treated with hemodialysis, vasopressors with Swan-Ganz monitoring, chloramphenicol, and methylprednisolone, but died on the tenth day of illness.

In view of the skin biopsy finding, the gallbladder and appendix were examined by direct immunofluorescence and revealed R. rickettsii in the walls of the blood vessels in each organ. Rickettsial vasculitis was observed in each layer from the lamina propria to the subserosa of each organ.

A 71 year old woman from eastern Tennessee developed headache, nausea, and vomiting for 2-3 days. She had a fever, right costovertebral angle tenderness, and pyuria. Upon refusing hospitalization, she was treated with cefamandole nafate and trimethoprim-sulfamethoxazole for presumed pyelonephritis. Two days later she was admitted with nausea, vomiting, weakness, and dehydration. Laboratory data included severe thrombocyutopenia, elevated liver enzymes, creatinine, and mild hypoxemia on oxygen therapy. She developed noncardiogenic pulmonary edema on her second hospital day. Ultrasonography of the right upper quadrant of the abdomen revealed a normal liver and a thickened wall of the gallbladder, surrounded by a sonolucent zone that was interpreted as a pericholecystic abscess or edema. Because of suspected pericholecystic abscess, exploratory laparotomy was performed on the second hospital day with cholecystectomy, intraoperative cholangiogram, and liver biopsy. The gallbladder contained no stones, and the cholangiogram was normal. There were 500-1000 ml of bile-stained ascites. Her postoperative course was characterized by persistent fever, respiratory failure, metabolic acidosis, renal failure, and cutaneous hemorrhages. Prior to laparotomy she was treated with various antibiotic regimens which included ampicillin, doxycycline, gentamicin, cefoxitin, piperacillin, and clindamycin. Doxycycline, an active antirickettsial drug, was given for less than a day. Postoperatively, she received gentamicin and chloramphenicol. Although the diagnosis of RMSF was considered during the illness, it was not made. She died on her sixth day of hospitalization.

Direct immunofluorescent examination of the surgically removed gallbladder and postmortem skin revealed R. rickettsii in blood vessel walls. Rickettsial vascular injury was observed.

Case 3 was a 55 year old man who developed fever, headache, and myalgia, six days before death. Over the period 2-4 days prior to death, he had intermittent nausea and vomiting. One day before his demise, he vomited a large blood clot and was hospitalized. Hematemesis continued requiring transfusion of six units of red blood cells. After transfer to Chapel Hill he had hematocrit 37%, platelets 18,000/ul, acute renal failure, seizures, cardiopulmonary arrest, hypotension, and postarrest hematocrit 19.5%. He was transfused with red blood cells and platelets. Endoscopy revealed a massive amount of blood in the stomach and esophagus, but no discrete bleeding focus. Despite immediate treatment with choramphenicol, the patient died one day after admission. Autopsy demonstrated gastric rickettsial vascular infection and injury with hemorrhage, the immediate cause of death.

Case 4 was a 4 year old girl in the preantibiotic era, 1933, in fact. Postmortem examination revealed, in addition to the typical findings of RMSF, a perforated appendix, the wall of which contained numerous injured blood vessels infected by immunfluorescent R. rickettsii. This case illus-

trates that R. rickettsii can actually cause lesions requiring surgical intervention.

Case 5 was a 10 year old male who developed fever, nausea, vomiting, and abdominal pain leading to appendectomy. Seven days later a history of tick bite was obtained and a rash was noted and demonstrated upon biopsy to contain immunofluorescent R. rickettsii. The hospital course was complicated by coma, seizures, acute renal failure, noncardiogenic pulmonary edema, cardiopulmonary arrest, skin necrosis, and severe thrombocytopenia, but the patient survived and was discharged from the hospital after more than a month with convalescent IFA of 1:8,192. Review of the resected appendix did not reveal any rickettsial vascular injury or immunofluorescent R. rickettsii. Thus, our dilemma is that RMSF may be misdiagnosed as acute surgical abdomen or gastoenteritis without a crucial intraabdominal lesion that would benefit from surgery or R. rickettsii-associated lesions may cause fatal gastrointestinal hemorrhage or cause life threatening gastrointestinal lesions such as ruptured appendix, a lesion requiring surgical intervention. Finally, although we believe the g.i. manifestations of RMSF are associated with the abdominal rickettsial vasculitis, demonstrable rickettsial infection and lesions may not always be present in the tissue examined, particularly a surgical specimen.

A manuscript on the acute phase reaction in boutonneuse fever has been prepared and submitted for publication. This work was performed in Palermo in collaboration with the Clinic of Tropical and Subtropical Diseases and the Institute of Hygiene. Evaluation of specific serum proteins from 44 patients in Sicily with boutonneuse fever revealed that C-reactive protein, haptoglobin, alpha-1-antitrypsin, C3, and C4 are elevated at the time of presentation for medical attention in varying proportions of the patients. C-reactive protein is invariably elevated as a consequence of active injury and inflammation during the first week of illness. These increased serum protein concentrations follow the pattern of the acute phase reaction and suggest that immunopathologic phenomena and intravascular hemolysis do not occur in most patients with boutonneuse fever.

The haptoglobin concentration was significantly higher during the first two weeks of illness than subsequently. Only three determinations had values below the normal range, one during the first week of illness and two during late convalescence. Serum alpha-1-antitrypsin levels were highest during the first two weeks of illness and had diministhed to normal levels by 4 weeks after onset of illness. Concentrations of C3 convertase and C4 were elevated acutely with 16/25 measurements of C4 and 8/25 measurements of C3 convertase above the reference interval during the first week and 20/26 assays of C4 and 5/26 assays of C3 convertase above the reference interval during the second week. In only one patient during the first week of illness were the concentrations of C3 convertase and C4 below the reference interval. C-reactive protein (CRP) was at or above the upper limit of normal in all patients during the first week and in 20/26 patients during the second week. By late convalescence 1-2 months after onset, 15/20 patients had no detectible serum CRP.

This study has demonstrated that the acute phase reaction ocurs during boutonneuse fever. Serum concentrations of certain hepatic synthesized proteins are elevated as a sterotyped response following surgical operation, several acute infections, and other situations that have acute inflammation or tissue necrosis. CRP concentration has been shown to rise from undetectible levels within 4-6 hours after acute injury. Haptoglobin, fibrinogen, and alpha-1-antitrypsin are increased in concentration by 24 hours after injury, and C3 convertase is elevated during the first week. Our measurements were

not made early enough in the course of BF to determine how many hours after onset were required before the rise occurred.

The effects of the elevated concentrations of these serum proteins on the pathophysiology of BF is uncertain. It may be hypothesized that C3 convertase and C4 might be consumed by binding to circulating immune complexes, which have been demonstrated to occur in BF. Although the observation that C3 convertase and C4 concentrations are mostly elevated or normal does not exclude that possiblity entirely, it does not support that hypothesis. Other observations and experimental data suggest that immunopathologic mechanisms are not important in spotted fever group rickettsioses (Bradford et al, 1979; Jerrells and Eisemann, 1983; Kenyon and Pedersen, 1980; Kokorin et al, 1976; Montenegro et al, in press; Moser et al, 1977; Walker and Henderson, 1978). Nonspecific opsonization of spotted fever rickettsiae by direct reaction with components of complement or CRP has never been demonstrated; however, elevated concentrations, particularly of CRP, suggest this as a possible host defense mechanism as it may be in other bacterial infections. The persistent elevation of CRP in some patients indicates that tissue injury or inflammation is ongoing.

The observation of a low plasma concentration of haptoglobin in one patient during the acute phase of BF indicates that significant hemolysis is not a frequently occurring complication. The recent demonstration of hemolysis in a patient with glucose-6-phosphate dehydrogenase (G6PD) deficiency and fatal RMSF suggests that hemolysis may play a role in the enhanced severity of RMSF in G6PD deficient men (Walker et al, 1983). Since males with G6PD Mediterranean have been shown to have a higher incidence of specific complications in BF than G6PD normal males (Piras et al, 1983), the possibility of hemolysis should be now evaluated in this subpopulation of patients with BF.

In general, the acute phase reaction appears to be an immediate response which precedes the generation of a specific immune response. Like fever, this response is highly likely to have resulted from evolutionary selection and to have an effect that is generally favorable to the host. The exact interactions of C3 convertase, C4, and CRP with R. conorii await further study.

A manuscript on familial cases of boutonneuse fever has been prepared and submitted for publication in collaboration with the same groups of investigators in Sicily. Pairs of cases of boutonneuse fever occurred in three families. The illness appeared nearly simultaneously in both members of the family, but generally was more serious in one as judged by clinical and laboratory parameters. The possibility of a "bed rickettsiosis," that is, reactivation of rickettsiae by the blood meal obtained from the first individual by the same tick which fed upon the second individual, can be excluded in two of the three pairs of cases. Four of the six patients were sleeping in different beds.

On the other hand, the differing severity may be reflected in the different immunological patterns of the two patients. In one couple it was documented that the first and more seriously ill had antibodies of the IgM class, presumably a result of the first exposure to Rickettsia conorii. The second and less ill patient had antibodies of the IgG class only, presumably the result of reexposure after previous asymptomatic infection with a spotted fever group rickettsia.

The cases reported here, particularly the two sisters, comprise an interesting example from the point of view of clinical and laboratory observations. Clinically, the course was remarkable more severe, as judged by meningeal signs, and more extensive hemorrhagic rash, hepatomegaly,

lymphadenopathy, in the younger (54 year old) sister who did not have a signifícan prior medical history in comparison with her sister (74 year old with a four year history of hypertension). In general, rickettsioses have been documented to be more severe in older patients and clinical judgement would suggest in those with underlying medical problems. Also the laboratory data demonstrated more marked abnormalities in the first of the two patients to become ill.

The hypothesis that a single source of infection, that is to say the same tick, first bit the older sister and then the younger sister with progressive "reactivation" of the rickettsiae after the first blood meal of the tick does not seem very probable. This hypothesis has been proposed in several cases of Rocky Mountain spotted fever occurring in married couples, familial rickettsioses of the bed (Parker, 1933; Schaffner et al, 1965), but this proposition is not supported by our cases since the two sisters, although living in the same house, had separate bedrooms.

It seems to us instead more probable that the explanation has an immunologic basis supported by the course of development of immunofluorescent antibodies of a particular class. The younger sistar had predominantly IgM antibodies early in her course, and her sister had IgG antibodies. This suggests that the more severe case encountered infection with R. conorii for the first time while the milder case had an anamnestic immune response with rapid rise in the IgG class of antibodies, possibly an indication of previous asymptomatic infection with a spotted fever group rickettsia. This immediate immune response, therefore, allowed a faster antibody rise, and thus, better control of the pathogenic events. It is clear that this hypothesis goes beyond the documented evidence; however, we recently observed a typical "tache noire" (in which biopsy was demonstrated the presence of R. conorii by immunofluorescence) in a patient who did not develop subsequent illness. Meanwhile, monitoring of the immune response showed seroconversion of only IgG antibodies. This observation supports the hypothesis that preexisting asymptomatic infection allowed an anamnestic immune response sufficient to circumscribe the infection at the site of inoculation (Burgeois et al, 1982; Mansueto et al, 1984a).

Also in this report are two pairs of cases that appear to document a dynamic analogue. Indeed, in the subjects with a predominant initial IgG response the illness was less severe. The role of humoral immunity in the pathogenesis of rickettsial diseases is not completely clear; nevertheless, several experimental studies have demonstrated a role for antibodies in an eloquent manner. Gambrill and Wisseman have observed that human immune anti-R. prowazekii serum augments the opsonizing power and the destruction of rickettsiae by leukocytes and macrophages. Topping (1940, 1943) demonstrated protection by prophylactic administration of immune sera. The military significance of these observations is that there are probably "hot spots" of R. conorii prevalence in the ecosystem into which nonimmune troops might enter and become infected with a substantial attack rate for the group.

A manuscript has been prepared and submitted for publication on the epidemiology of boutonneuse fever in western Sicily that correlates the distribution and prevalence of R. conorii infection in Rhipicephalus sanguineus with canine seropositivity and human cases of boutonneuse fever.

The distribution and prevalence of spotted fever group rickettsial infection in the ixodid dog tick Rhipicephalus sanguineus were found to occur at a rate of 19.7% with variation related to geographical and socio-occupational factors. A higher rate of infection was demonstrated in ticks removed from dogs associated with documented cases of boutonneuse fever.

Out of the 1078 ticks removed from dogs, R. sanguineus was predominant with few other species (4 Ixodes ricinus, 4 Hyalomma marginatum, 2 R. bursa). All stages of development were obtained from sheep-dogs, while only adults were observed in domestic dogs living in urban areas and, in late summer, in dogs of the mountainous areas. Only adult ticks were tested in this study. In each place the occurrence of BF has been documented.

A total of 212 (19.2%) of 1078 ticks was found infected with SFG rickettsiae. Not all the areas investigated, however, showed the same rate of infection but a gradient was observed which increased from urban (Palermo, Trapani, Agrigento) to rural areas (Carini, Castellammare, Vicari, Santo Stefano di Quisquina, Alcamo) and decreased again with a rise in altitude (Petralia and the Madonie Mountains). Ticks obtained from dogs living in sheepfolds in rural areas frequently contained SFG rickettsiae; 23/24 (95.8%) of these dogs were found to harbour infected ticks, and all of them where seropositive. In urban areas 14 (43.7%) of 32 dogs were free of infected ticks ($P<0.01$) although antibodies reactive with R. conorii were detected in 25/32 (78%). Higher percentages of dogs carrying noninfected ticks and seronegative dogs were found in mountainous areas: 14/21 (67%) and 10/21 (47%), respectively ($P<0.001$). Seventy-three dogs (67.6%) were found to harbour infected ticks overall. Antibodies reactive with R. conorii were present in 88/108 (83%); yet only 20 of 35 (57.1%) dogs with uninfected ticks were seronegative ($P<0.001$).

Investigation of 8 human cases of BF where association with dogs was documented revealed that 16 out of 22 (73%) dogs tested harboured ticks infected with R. conorii as demonstrated by direct examination of the ticks and seroconversion of guinea pigs. All the dogs were found to have anti-R. conorii antibodies. It is intriguing to note that the case from Santo Stefano di Quisquina was a shepherd and the patients in Castellammare and Vicari, a school teacher and a grocer, respectively, had a sheepfold very near the land surrounding their homes. Controls were chosen and taken from the same neighborhood from houses where no cases of BF had occurred; a significantly lower percentage of seropositive dogs and infected ticks was observed in the controls ($P<0.001$). A tick (R. sanguineus) removed from the site of bite on the shoulder of an agricultural worker contained SFG rickettsiae demonstrated by microscopic observation and immunfluorescent staining of hemolymph; seroconversion occurred in the inoculated guinea pig. The dog owned by this patient was also seropositive. All guinea pigs inoculated with hemolymph positive ticks showed seroconversion after 28 days.

The essential role of the tick is emphasized by the high rate of tick infection (19.7%) and the uneven distribution of SFG rickettsial infection of ticks ranging from 4% to 35% in different areas. These variations correlated with geopgraphic factors: high infection rates in rural areas and lower altitudes and, conversely, lower infection rates in urban and mountainous areas.

These observations of high proportions of rickettsia-infected ticks and seropositive dogs in areas of Sicily with higher incidence of BF are similar to the evidence for greater rickettsial activity in dogs in France and the USA in areas of higher human attack rates for BF and Rocky Mountain spotted fever, respectively.

There was an even higher rate of SFG rickettsial infection of ticks from dogs that were associated with human cases of BF in Sicily than the average rate of tick infection; 73% of such dogs had SFG rickettsia-containing ticks. The stimulation of seroconversion of guinea pigs inoculated with tick-rickettsia suspensions strongly suggests that these SFG rickettsiae were, in fact, pathogenic R. conorii. This association of R.

conorii-infected R. sanguineus with human BF in western Sicily was most conclusively documented by the isolation of a SFG rickettsia from a R. sanguineus tick which was removed from a patient with BF.

A manuscript reporting four cases of boutonneuse fever that occurred during the cold season of the year has been prepared and submitted for publication in collaboration with investigators at the University of Palermo and Dr. Raoult of Marseille. These documented cases occurred in Sicily and France between November and February. It cannot, therefore, be assumed that boutonneuse fever is limited to the warm weather seasons of the year. The diagnosis must be considered for febrile patients in the Mediterranean basin throughout the year.

A manuscript that documents the occurrence of hemolysis and severe boutonneuse fever in a previously healthy 27 year old man with glucose-6-phosphate dehydrogenase deficiency has been prepared and submitted for publication in collaboration with the Infectious Diseases Unit of the Boigny Hospital and the Pediatric Hematology Unit of La Timone Hospital in Marseille. A 27 year old previously healthy Algerian man was hospitalized on August 3, 1984, after two days of fever, headache, myalgias, asthenia and weight loss of two kilograms. Several days before hand he had noticed a scrotal lesion with painful inguinal lymphadenopathy. On admission he had temperature 39.5°C, pulse 100/min, blood pressure 110/60 mmHg, and a scrotal eschar. Laboratory data included normal urinalysis, hematocrit 45%, erythocyte count 4.6 x 10^6/ul, hemoglobin 14.65 g/dl, white blood cells 8.4 x 10^3/ul, with 77% polymorphonuclear leukocytes, platelet count 128 x 10^3/ul, serum sodium 137 mmol/l, serum potassium 3.7 mmol/l, serum chloride 99 mmol/l, serum calcium 2.4 mmol/l, prothrombin time 11 seconds (control 12 seconds), unconjugated bilirubin 11 mc mol/l, aspartate aminotransferase (SGOT) 25 IU/l, and blood urea nitrogen 5.1 mmol/l. He became stuporous and confused. A maculopapular eruption involving the palms and soles and purpuric on the legs and trunk was observed on August 7, 1984. The diagnosis of Mediterranean spotted fever was made, and intravenous treatment with doxycycline, 200 mg/day, was begun on August 7. On August 10, 1984, the patient was asthenic and stuporous but apyretic. Laboratory data showed hepatic involvement (SGOT, 145 IU/l and SGPT, 142 IU/l) and evidence of hemolysis with decreased hemoglobin and haptoglobin but normal bilirubin. The subsequent course was uneventful and did not require blood transfusions. G6PD phenotype was determined to be G6PD B⁻ by low G6PD activity determined by spectrophotometry and by electrophoretic mobility. The diagnosis of Mediterranean spotted fever was confirmed by immunofluorescent antibody test. The investigation of the family revealed two other cases of G6PD deficiency, a brother and a maternal uncle.

Severe cases of Mediterranean spotted fever have been reported previously. These cases were usually males, with underlying diseases such as diabetes, cardiac insufficiency, or alcoholism. G6PD deficiency was suspected to be one of the risk factors for severe disease. G6PD deficiency is rare in France, but in the southern France especially in Marseille, a population of diverse origins is present. In Marseille 10% of the population originally came from Corsica and 18% from North Africa where G6PD deficiency is prevalent. The rate of G6PD deficiency is 10% in Algeria. G6PD deficiency has previously been associated with severity in murine typhus, scrub typhus and Rocky Mountain spotted fever. Regarding Mediterranean spotted fever, Meloni and Forteleoni found no statistical differences in patients with and without G6PD deficiency although no particular criteria that were evaluated were stated. In contrast, Piras et al reported an evaluation of Sardinian males with Mediterranean spotted fever

for complications of particular organ systems which occured more frequently in G6PD deficient patients. Neither study commented on examination for hemolysis.

Our patient had a quite severe form of serologically confirmed Mediterranean spotted fever with stupor, confusion, purpuric exanthem thrombocytopenia, hepatic involvement, and evidence for hemolysis. The hemoglobin fell from 14.7 g/dl to 9.9 g/dl, and serum haptoglobin was depleted (0.16 g/l) acutely and rose to normal concentration (1.5 g/l) during convalescence. This is the first reported case of hemolysis in a G6PD deficient patient suffering from Mediterranean spotted fever and the first reported case of hemolysis with rickettsiosis in a G6PD B⁻ (Mediterranean) patient. The mechanism of enhanced severity of rickettsioses in G6PD deficiency is unclear. Two hypotheses are drug induced hemolysis and increase of rickettsial activity due to defective host defense or stimulation by constitutents released from lysis of red blood cells. In our case hemolysis occurred before tetracycline treatment. In our experience this man was the only patient with documented G6PD deficiency among 49 males with Mediterranean spotted fever where the G6PD activity was determined. Furthermore, he is the only patient under 30 years of age to have a severe form of the disease. In our opinion, in addition to alcoholism, diabetes and old age, G6PD deficiency could be considered as a cause of enhanced severity in Mediterranean spotted fever as well as in other rickettsioses.

This report suggests that servicemen, particularly those of African and Mediterranean ancestry, should be evaluated for glucose-6-phosphate dehydrogenase phenotype before assignment to duty in southern Europe, Africa, or the Middle East. Documentation of this genetic condition may allow particular preventive measures to be taken, e.g. vaccination, if it becomes available, or anticipation of severe disease if boutonneuse fever is not diagnosed and treated early in the course.

Other investigations involving study of human rickettsioses that were subjects of work during the past year include the publication of the article, "Correlation of the Distribution of Rickettsia conorii, Microscopic Lesions, and Clinical Features in South African Tick Bite Fever" in collaboration with J.H.S. Gear, and autopsy case study of a fatal case of boutonneuse fever from Salamanca, a study of a rache noire biopsy from a case of boutonneuse fever in a tourist who returned from the Mediterranean region to San Franscisco, and investigation of fatal cases of epidemic typhus in North Africa during World War II. The South African case, which was described in last year's annual report, was completed and presented as a paper at the annual meeting of the American Society of Tropical Medicine and Hygiene and an original research article in the Journal of the American Society of Tropical Medicine and Hygiene (see enclosed reprints). The fatal case from Salamanca is currently being prepared for immunofluorescent demonstration of R. conorii. Antigen of R. conorii was detected in sparse quantity in the tache noire sent from San Fransisco. The method for demonstration of R. prowazekii in fixed human tissues that have been stored embedded in paraffin since the early 1940's has been developed using a conjugate reactive with typhus group rickettsiae that was obtained from the CDC. Tissues of three cases from the AFIP archives are in the process of preparation in our laboratory for a clinicopathologic study in collaboration with Dr. Ted Woodward. Preliminary results of sensitive, immunofluorescent examination for the distribution of R. prowazekii have shown rickettsiae in endothelial locations in kidney and heart. We hope to elucidate several pathophysiologic aspects of epidemic typhus and to compare the distribution

of rickettsiae with RMSF and boutonneuse fever.

Our work on the mouse model of R. conorii infection that was begun prior to the initiation of this contract was prepared as a completed manuscript and published this year (Montenegro, M.R., Walker, D.H., Hegarty, B.C., Infection of genetically immunodeficient mice with Rickettsia conorii. Acta Virol 28:508-514, 1984). It includes descriptions of a model of R. conorii hepatitis that is analogous to human boutonneuse fever hepatic lesions, particularly as it occurs in immunocompetent mice. Work is in progress toward further elucidation of hepatic injury by R. conorii and the local immune response to hepatic rickettsial infection by electron microscopy and immunohistochemistry. A good animal model for documented, reproducible severe R. conorii infection of organs pertinent to severe human infection other than liver has not been described and must be developed. Study of C3H and Balb/c mice with R. conorii inoculated by intraperitoneal, subcutaneous, or intradermal routes has been conducted in collaboration with Dr. Tom Jerrells at Walter Reed Army Institute of Research. We have examined by light microscopy the tissues of mice susceptible (C3H) and resistant (Balb/c) to lethal R. conorii infection by the intraperitoneal (lethal for C3H), subcutaneous (resistant in both mouse strains), and intradermal (resistant in both mouse strains) routes over the first 12 days after inoculation. Lesions are consistently present in hepatic tissues of these mice (Table 5). Renal and CNS lesions are less consistent. Immunofluorescent determination of the presence, distribution, and relative quantity of rickettsiae in these tissues by the deparaffinization, trypsin digestion method revealed that rikettsiae were present in the peritoneum and liver of lethally infected CBH mice inoculated intraperitoneally when sacrificed on day 7. Systemic endothelial infection was not observed in any of the mice. The lethal combination is, like the other combinations, therefore, neither an acceptable model of R. conorii endothelial injury nor of immunity to endothelial infection.

During the period of a visiting professorship by Drs. Mansueto and Tringali in March of 1985, tissues of guinea pigs inoculated with R. conorii were examined for lesions and immunofluorescent rickettsiae. Leisons were few and inconsistently observed except for periorchitis and multifocal hepatic inflammation. Immunofluorescent R. conorii were detected in the mesothelium of the tunica vaginalis but not in systemic and pulmonary vascular endothelium. Thus, guinea pigs, in which even fever is frequently absent or of short duration, are not a model for study of the pathophysiology and pathogenesis of R. conorii infection.

Because cotton rats have been reported as models for several human viral infections and R. prowazekii infection and latency, this species, Sigmodon hispidus, was inoculated with R. conorii, R. sibirica, and R. rickettsii. The results were disappointing with no deaths and few lesions or demonstrated SFG rickettsiae after an extensive histologic and immunofluorescent examination.

Collaborative assistance has been provided to Dr. T. S. Walker in probing the role of prostaglandins in the pathogenesis of SFG rickettsioses. Plasma and sera from experimentally infected guinea pigs and human cases have been collected and sent to this laboratory for analysis. The results are pending at this time.

Investigations of cell culture models of R. conorii injury included final preparation of the manuscript and publication of the article "Injury restricted to cells infected with spotted fever group rickettsiae in parabiotic chambers," Acta Tropica, 41:307-312, 1984, by Walker, D. H., Firth, W. T., Hegarty, B. C., previously reported in annual report number 1

(reprints enclosed), evaluation of the effect of temperature on cell injury by R. conorii, and work toward development of a model of cell injury with fluid overlay by measurement to lactate dehydrogenase concentration in culture medium. Plaques were not appreciably different in quantity at 32°, 34° and 36°C. At 38°C fever R. conorii plaques were observed. The LDH model has been characterized in that predominantly the isoenzyme LDH-5 is contained in and released by Vero cells. An assay that preferentially measures this isoenzyme is under investigation. The major problem at present relates to the relatively small amount of LDH released and standardization of a protoocol for precision in determining the concentration in the medium. The model is not currently ready for use in pursuing the mechanisms of cell injury related to phospholipase A and trypsin-like protease.

Some effort has been expanded on review and analysis of the published medical and scientific literature on rickettsial disease problems. This effort has been useful in focusing on the critical issues that these diseases pose. A major product of this work is the writing of a comprehensive chapter for a CRC Handbook on viral hemorrhagic fevers and rickettsial diseases edited by Dr. J.H.S. Gear. The chapter entitled "Pathology and Pathogenesis of the Hemorrhagic State in Viral Hemorrhagic Fevers and Rickettsial Diseases" encompasses correlation of clinical and hemorrhagic manifestations, human and experimental animal pathologic lesions, and investigations of hemostatic and pathogenic mechanisms in these diseases. A second major work on synthesis of the current state of knowledge is the planning and editing of a CRC Handbook, Biology of Rickettsial Diseases. It is my goal to produce a comprehensive work to encompass the meaning of the recent advances in rickettsiology and their implications for understanding rickettsial ecology, physiology, immunity, pathology, and clinical diseases. The field lacks a book that presents its interesting problems as they currently exist in a form that transcends the individual article or collection of research publications. The results should be useful for military medical planning and will include contributions from rickettsiologists with a perspective of rickettsiae that include military significance. Drs. Gregory Dasch, Thomas Jerrells, Jim Williams, Emilio Weiss, and I are among the authors of the various chapters.

Table 1

Evaluation of Taches Noires from Sicilian Patients with Confirmed *Rickettsia conorii* Infection

Patient	Cutaneous Necrosis	Vasculitis	Thrombosis	Dermal Edema	Predominant[a] WBC	IF[b] R. conorii
1	+++[c]	+++	+	++	L,M	+
2	0	+++	0	++	L,E	+
3	+	+++	0	++	L,M	+
4	++	+++	++	++	L,M	+
5	0	+++	+	++	L,M	+
6	0	++	0	++	L	0
7	0	++	+	+	L	+
8	+++	++	+	++	L	+
9	0	++	0	++	L	0
10	+++	++	0	++	L	0
11	+++	+++	0	++	M	0
13	+++	+++	+++	++	L,P	+
14	+++	+++	0	+	L	+
16	+++	+++	0	+	L	+
17	NE[d]	++	0	++	L	+
18	+++	+++	0	+	M,L	+

a - Predominant perivascular leukocytes: L, small lymphocyte; M, large mononuclear cell: E, eosinophil; P, PMN
b - Presence (+) or absence (-) of immunofluorescent *Rickettsia conorii*
c - Absent (0): +, mild; ++, moderate; +++, severe
d - Feature not available for evaluation

Table 2.
Clinical and Serologic Data for Sicilian Patients
with Boutonneuse Fever Undergoing Liver Biopsy

Patient	Age/Sex	Tache noire	Rash	Fever	Serology	
					Acute	Convalescent
1	43M	+[a]	0[b]	+	160[c]	N.D.[d]
2	74M	+	+	+	160	160
3	66F	+	+	+	40	N.D.
4	67F	+	+	+	N.D.	320
5	86F	+	+	+	40	160
6	68M	+	0	+	40	N.D.
7	50F	+	+	+	320	1280

a - present
b - absent
c - reciprocal of indirect immunofluorescent antibody titer against R. conorii
d - not determined

Table 3.
Evaluation of Sicilian Patients with Boutonneuse Fever for Hepatic Involvement

Patient	LDH[a]	ALT[b]	AST[c]	AP[d]	GGT[e]	Bil[f]	day of bx[g]	Lesions	IF R. conorii
1	120	15	22	139	15	normal	1	+[h]	0[i]
2	259	16	20	N.D.	51	normal	3	+	0
3	390	38	30	152	normal	1.1	6	+	0
4	251	10	14	N.D.	N.D.	0.9	13	+	0
5	255	28	18	290	18	normal	2	+	0
6	210	21	18	199	N.D.	normal	2	+	0
7	N.D.	85	93	477	160	1.0	30	+	0

a - serum lactate dehydrogenase (reference interval, 100-240 IU/l)
b - serum alanine aminotransferase (reference interval, 6-31 IU/l)
c - serum aspartate aminotransferase (reference interval, 6-31 IU/l)
d - serum alkaline phosphatase (reference interval, 60-170 UI/l)
e - serum gamma glutamyl transpeptidase (reference interval, 4-18 IU/l)
f - total serum bilirubin (reference interval, less than 1.0 mg/dl)
g - number of days after onset of fever
h - presence of hepatic lesions
i - absence of immunofluorescent R. conorii

Table 4.

TABLE: Patients Reported with Rocky Mountain spotted Fever Leading to Laparotomy

Patient	Age/ Sex	Preoperative Abdominal Symptoms and Signs	Organ Removed	Rickettsiae	Vascular Lesions	Laboratory Diagnosis	Ref.
1	16M	RLQ abdominal pain, nausea, diarrhea, vomiting, RLQ tenderness	Appendix	N.R.*	+	Weil Felix 1:640	3
2	17F	Abdominal pain, nausea, vomiting, rebound tenderness	Gallbladder	N.R.	N.R.	Complement fixation	5
3	9F	Abdominal pain, diarrhea, RLQ mass	Appendix	N.R.	N.R.	N.R.	8
4	18M	RLQ abdominal pain and tenderness, hyperactive bowel sounds	Appendix	N.R.	N.R.	DFA† demonstration of R. rickettsii at necropsy, Isolation of R. rickettsii	8
5	6½M	Nausea, vomiting, abdominal pain, guarding and rebound tenderness	Terminal ileum	+ (P.S.)‡	+	Weil Felix 1:40 at first hospital rising to 1:320 at second hospital	9
6	60M	LLQ abdominal pain and tenderness, hyperactive bowel sounds	Appendix	+ (DFA)	+	DFA, IHA1:1024,§ Weil Felix OX19 negative rising to 1:320, OX 2 1:40 rising to 1:320	10
7	76M	Right abdominal pain	Gallbladder Appendix	+ (DFA) + (DFA)	+	DFA of skin, appendix, and gallbladder	Present report
8	71F	Nausea, vomiting	Gallbladder	+ (DFA)	+	DFA of skin and gallbladder	Present report

*N.R. - not reported
†DFA - direct fluorescent antibody demonstration of R. rickettsii
‡P.S. - Pinkerton's stain
§IHA - indirect hamagglutination assay

Table 5.

Evaluation of Susceptible and Resistant Mice for Lesions after Inoculation with *Rickettsia conorii*

mouse strain	route	lethality	da. of sac.	Lesions in Liver	Brain	Kidney
C3H	IP	+	7	4/4	1/4	0/4
Balb/c	IP	0	7	2/2	0/2	0/2
C3H	SQ	0	7	2/2	0/2	0/1
C3H	SQ	0	10	0/1	0/2	0/2
Balb/c	SQ	0	12	1/1	0/1	1/1
Balb/c	SQ	0	7	2/2	0/2	0/2
Balb/c	SQ	0	10	1/2	0/2	0/2
C3H	ID	0	12	1/1	0/1	0/1
C3H	ID	0	7	1/1	1/2	2/2
C3H	ID	0	10	3/3	2/3	2/3
C3H	ID	0	11	1/1	1/1	1/1
Balb/c	ID	0	12	1/1	1/1	1/1
Balb/c	ID	0	7	2/2	0/2	2/2
Balb/c	ID	0	10	3/3	0/3	0/3
Balb/c	ID	0	11	2/2	0/2	1/2
			12	1/1	0/1	0/1

Conclusions:

The currently recognized high rate of R. conorii infections in Spain, Portugal, France, Israel, and Italy is likely to continue. The absence of data from northern, eastern, and western Africa and the Middle East reflect the failure of clinical and epidemiologic systems in these areas to focus the necessary laboratory methods and organizational efforts on the problem. The disease is likely to be or to become an important unrecognized cause of incapacitating febrile illness in these areas. Boutonneuse fever is a neglected disease that has recently attracted investigation in Sicily, Marseille, and Salamanca. Collaborative relationships with these laboratories encourages study of the epidemiologic, clinical, pathophysiologic, and rickettsiologic questions at a admirable level of effort and expertise. This contract has fostered a significant part of this result and can continue to play an important role in maintaining enthusiasm for elucidating the unknown factors that boutonneuse fever encompasses.

It is probable that R. conorii infections are a greater cause of morbidity and mortality than is now appreciated. In Marseille, Salamanca, Israel, and South Africa, severe illness is regularly recognized in a substantial portion of those affected. The application of immunofluorescence to autopsy specimens is revealing the rickettsial and clinicopathologic basis for the pathophysiologic observations such as meningoencephalitis, hepatitis, and other visceral involvement. The documentation of hepatic lesions in seven consecutive Sicilian patients with hepatic biopsies during boutonneuse fever demonstrates the serious nature of the disease. The recognition that boutonneuse fever may cause an incapacitating febrile illness with neither rash nor tache noire suggests that the high incidence of antibodies to R. conorii in various populations may very well be due to prior undiagnosed symptomatic R. conorii infection. Specific dignostic tests are not applied to patients who do not present at least some of the classic manifestations of the disease, particularly a rash. Such a situation is obviously a potential threat to military health of soldiers in the wide geographic distribution of R. conorii. This situation is analogous to the problem of scrub typhus in World War II, e.g. the Assam-Burma theater of operations.

Particular problems that have been identified recently are the frequent hepatic involvement, lifethreatening gastrointestinal hemorrhage, severity of illness in glucose-6-phosphate dehydrogenase deficient men even if they are young and previously healthy, the occurrence of cases even in cold weather months, and occurrence of familial cases that suggest "hot spots" of R. conorii endemicity.

Our careful study of the tache noires has elucidated important factors in the pathogenesis that conform to current clinical features of R. conorii pathogenesis: 1) R. conorii is present in the lesions and appears to play a direct cytopathic role in vascular injury, 2) vasculopathic edema is a major pathophysiologic effect, 3) ischemic necrosis is not necessarily the end result of vascular injury, 4) thrombosis is not the major pathologic effect of vascular injury and, thus, should not be treated with anticoagulation, and 5) in human disease the local antirickettsial immune and inflammatory response is predominantly lymphocytes and macrophages.

Electron microscopy and immunofluorescence have not yet demonstrated large quantities of R. conorii in tache noires or liver biopsies. The possible explanations include: 1) the host immune and phagocytic response in these lesions may be effective at the stage of illness when patients are referred for tertiary subspecialty medical care, 2) antirickettsial anti-

microbial therapy may have been administered prior to collection of specimens, 3) ultrastructural sampling may be a problem. Planned approaches to this problem are attempts to obtain biopsies from patients earlier in the course and assignment of laboratory personnel to the task of greater effort at examining samples of the biopsies by electron microscopy and utilization of deparaffinization electron microscopy of foci in paraffin blocks that are demonstrated to contain R. conorii by immunofluorescence. More skin punches have been distributed to peripheral collaborative sites and more collaborative institutions have been recruited.

Mouse R. conorii hepatitis is a good model for human boutonneuse fever hepatic lesions. The mouse model should be examined to determine the target cell type and host immune response by immunohistochemistry and electron microscopy for endothelial cells, B-cells, T-cell subsets, and macrophages. The target cell of immunodeficient murine R. conorii hepatitis is a good candidate for in vivo ultrastructural evaluation of the relationship of rickettsial quantity to cytopathology and the ultrastructural features of cytopathology.

Other mouse models of SFG rickettsial infection must be evaluated for a model of SFG endothelial infection and injury in order to correctly investigate pathogenesis, pathophysiology, and immunity.

Monoclonal antibodies to R. conorii have been developed under a separate grant from the National Institute of Allergy and Infectious diseases and will be characterized as to their molecular targets as a function of the purposes of that research project on rickettsial antigens and vaccine development. These monoclonals should be used to study pathogenic mechanism of R. conorii under this Army contract in cell cultrue and animal models. The NIH grant does not have the funds or goals to support investigation of specific blocking of pathogenic mechanism(s). However, if pathogenic mechanisms that may be blocked by monoclonal antibodies are discovered, the most effective approaches to prevention and adjunctive treatment may be developed.

Recommendations:

1. Offer skin biopsy immunofluorescent demonstration of R. conorii in tache noire as a reference military laboratory dignostic test for boutonneuse fever.

2. Study the rickettsial hepatitis of boutonneuse fever and experimental R. conorii infection as models for visceral tissue injury by R. conorii.

3. Initiate a search for nonpathogenic R. conorii or R. conorii-like isolates in ticks in Sicily.

4. Develop a more rapid, quantifiable assay system for growth of and cell injury by R. conorii for screening amidine type trypsin-like protease inhibitors as potential prophylactic or adjunctive therapeutic agents.

5. Screen amidine type protease inhibitors for inhibition of cell injury by R. conorii.

6. Continue studies of tache noires for role of thrombosis in cell injury and specific identification of local immune cell types.

7. Continue in vitro study of pathogenic mechanisms of cell injury by R.

conorii.

8. Initiate epidemiologic surveillance of troops in Spain, Italy, and other places in the geographic distribution of R. conorii for acute cases of boutonneuse fever and for seroprevalence of of antibodies to R. conorii.

9. Obtain seroepidemiologic data on the prevalence of R. conorii infection in indigenous populations in Africa and the Middle East.

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Injury restricted to cells infected with spotted fever group rickettsiae in parabiotic chambers

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Summary

One chamber of paired parabiotic chambers separated by 0.2 μm pore-sized membrane filters which prevented passage of rickettsiae were infected with either *Rickettsia rickettsii* or *R. conorii*. Infected VERO cell monolayers underwent necrosis. Uninfected monolayers in adjoining chambers which shared the same extracellular milieu including soluble rickettsial products did not undergo necrosis.

Key words: *Rickettsia rickettsii; Rickettsia conorii;* rickettsial pathogenesis; parabiotic chamber.

Introduction

The hypothesis that cell and tissue injury are mediated by a rickettsial toxin has been suggested (Moe et al., 1976; Murray, 1980) although an exotoxin has never been demonstrated and rickettsial lipopolysaccharides do not have potent toxic activity (Schramek et al., 1977). Much of the confusion concerning rickettsial pathogenesis is the result of the name given to the phenomenon of the lethal effect of large doses of viable rickettsiae when inoculated intravenously into mice (Bell and Pickens, 1953; Gildenmeister and Haagen, 1940). Traditionally, this rickettsial laboratory assay has been termed the "mouse toxin phenomenon" although it cannot be produced by rickettsiae that are metabolically inactive or dead (Bovarnick and Allen, 1954 and 1957), and this toxicity has never been produced by a purified component of rickettsiae.

Recent investigations by Winkler and coworkers and in our laboratory have suggested that a rickettsial enzyme may mediate rickettsial injury to host

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cell membranes. Phospholipase activity appears to play an important role in *Rickettsia prowazekii*-induced hemolysis (Winkler and Miller, 1980), in the immediate cytotoxicity of a large inoculum of *R. prowazekii* on cell monolayers (Winkler and Miller, 1982), and in plaque formation by *R. rickettsii* (Walker et al., 1983). Phospholipase activity is a plausible hypothesis for explanation of the mouse toxicity phenomenon. In this study parabiotic chambers were employed to determine whether any soluble rickettsial product would injure uninfected cells sharing the same culture medium with cells infected and killed by *R. rickettsii*.

Materials and Methods

Twenty pairs of sterile parabiotic chambers (Bellco Glass, Vineland, NJ) were separated by 25 mm diameter cellulose triacetate membrane filters (Gelman Sciences, Ann Arbor, MI) with 0.2 μm pore size sealed between the chambers with silicone stopcock grease. Coverslips measuring 10.5 × 35 mm were placed in each chamber and were seeded with 5×10^5 VERO cells (CDC Tissue Culture Unit, Atlanta, GA). After incubation at 37° C in minimum essential medium with 5% heat-inactivated fetal calf serum and 10% tryptose phosphate broth for 24–48 h, monolayers were confluent. The medium was removed, and 11–40 plaque-forming units of *R. rickettsii* (Sheila Smith strain) were inoculated into one chamber of each of 13 pairs of parabiotic chambers. After 30–45 min for adsorption of inoculum, 10 ml of the same medium was added. Five pairs of chambers were not inoculated with rickettsiae. Coverslips from adjoining inoculated and uninoculated chambers were examined for evidence of cell death as determined by trypan blue staining (Garvey et al., 1977) on days 3, 4, 5, 6 and 7 postinoculation and for presence and distribution of *R. rickettsii* by direct immunofluorescence (Walker and Cain, 1980) on days 5, 6, 7 and 9 postinoculation. Uninoculated pairs of chambers were examined as controls on day 7 after inoculation. For a positive toxin control, one chamber of each of two pairs was inoculated with a fresh clinical isolate of *Pseudomonas aeruginosa* with examination of chambers on day 3 and on day 5 by trypan blue staining. In a subsequent experiment, 36–360 plaque forming units of *R. conorii* (strain 7) were inoculated in a similar manner into one of the matched pairs of parabiotic chambers containing coverslips with monolayers of VERO cells. The coverslips were examined on days 5 and 6 by phase contrast microscopy after trypan blue staining and then after acetone fixation by direct immunofluorescence for rickettsiae.

Results

By day 3 after inoculation, foci of trypan blue-stained necrotic cells were present in the parabiotic chamber inoculated with *R. rickettsii*. Over the succeeding days, the trypan blue-stained foci appeared to enlarge progressively. By day 5 these foci consisted of 25–50 necrotic cells admixed with an equivalent quantity of viable cells. By day 7 the infection had become confluent and contained a majority of necrotic cells. The uninoculated monolayers both in chambers adjoining infected cytopathic cells and in control chambers exhibited a similar appearance with only a few (< 1%), single, randomly distributed trypan blue-stained cells. The presence and distribution of *R. rickettsii* as determined by immunofluorescence correlated with the distribution of cell necrosis. On days 5 and 7, monolayers were examined by trypan blue staining and subse-

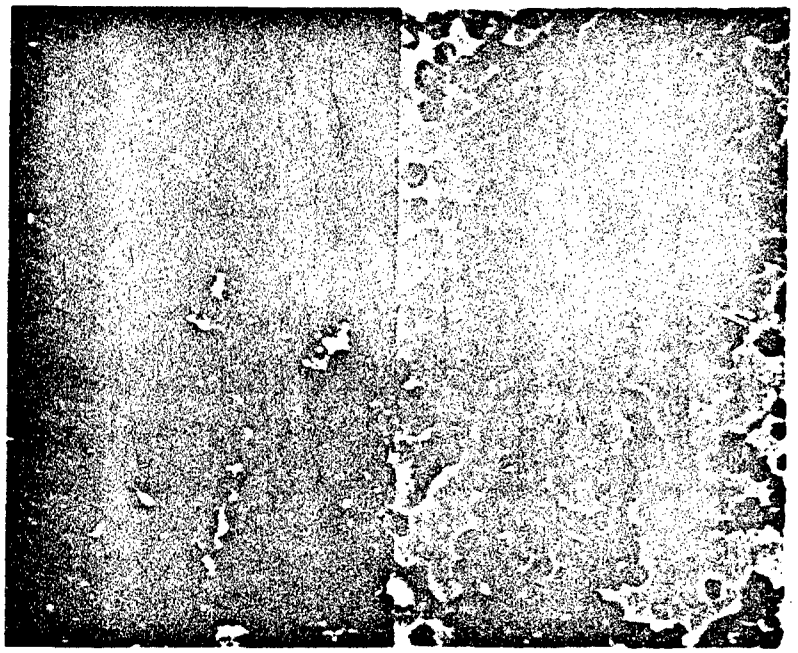

Fig. 1. Photomicrographs of the identical microscopic field of a monolayer in a parabiotic chamber 5 days after inoculation with *Rickettsia rickettsii*. Immunofluorescent demonstration of intense *R. rickettsii* infection in the lower portion of the field (left). Trypan blue-stained necrotic cells are numerous in the same area (right). FITC-labelled anti-*R. rickettsii* rabbit globulin (left) and phase contrast (right). × 300.

quent immunofluorescence on the same monolayer utilizing a microscope designed for both phase contrast and fluorescent microscopy. The areas of intense rickettsial infection and areas of trypan blue staining coincided (Fig. 1). No rickettsiae were detected in adjoining chambers that were separated by the 0.2 μm filter. The monolayers infected with *R. conorii* showed severe cytopathic effect but with less necrosis than *R. rickettsii* infected monolayers although nearly all of the cells were infected. In contrast, the adjacent uninoculated monolayers appeared without cytopathic effect. Validation of the parabiotic chamber toxin model was provided by demonstration of progressive destruction of the monolayers in the chamber infected with *P. aeruginosa* and in the uninfected chamber when examined on days 3 and 5.

Discussion

In this model of severe cell injury and cell death caused by spotted fever group rickettsiae, uninfected cells of the same type as those injured and killed by rickettsiae were exposed to the same extracellular milieu including concentration of soluble rickettsial products which would have passed freely along with other macromolecules through the 0.2 μm pore filter. None of these exposed yet uninfected cells exhibited any more cellular necrosis than control monolayers in which both parabiotic chambers were not infected with rickettsiae. These data argue strongly against the existence of an important rickettsial exotoxin or soluble enzyme analogous to the phospholipase of *Clostridium perfringens* in the pathogenesis of cell injury by *R. rickettsii*.

These data are compatible with the proposed phospholipase-associated penetration mechanism of cell injury by rickettsiae. *R. prowazekii* requires attachment to erythrocytes for accomplishing rickettsial hemolysis (Ramm and Winkler, 1973 and 1976; Winkler, 1977). *R. rickettsii* appears to require attachment to cells by the cholesterol-containing receptor in the plaque model to exert cell injury (Walker et al., 1983). Thus, prevention of passage of spotted fever group rickettsiae from one chamber to the adjoining chamber by the 0.2 μm pore-size filter would limit the phospholipase-associated penetration mechanism to the infected chamber. The more extensive frank necrosis with *R. rickettsii* infection than with *R. conorii* correlates with the greater incidence of complications and mortality in Rocky Mountain spotted fever than in boutonneuse fever.

This in vitro experiment does not exclude the possibility of a role for endotoxin in Rocky Mountain spotted fever although the data for a lipopolysaccharide with in vivo endotoxin activity for rickettsiae are weak. Classical endotoxin pathogenic mechanisms involve in vivo host-mediated mechanisms dependent on polymorphonuclear leukocytes and coagulation; these host-mediated pathogenic elements were not tested in this parabiotic chamber model.

This parabiotic chamber model was chosen over exposure of uninfected monolayers to filtered supernatant of rickettsia-infected cells. Since the parabiotic system compares the effect of the products of rickettsial infection over the same time course and concentrations as of the monolayer with rickettsial cytopathic effect, its negative results may be interpreted as valid. In contrast, acute exposure of monolayer to filtered supernatant would not reflect such a dynamic interaction.

The formation of enlarging foci of infection and necrosis occurs in this model with fluid medium that allows release of rickettsiae from infected cells into the medium and spread to infect randomly any cell of the monolayer. The contiguity of most infected and injured cells suggests that cell-to-cell spread of rickettsiae may be important in the pathogenesis of spotted fever group rickett-

sioses. This contiguous distribution of rickettsia-infected and injured cells is analogous to our observations of rickettsial distribution in human Rocky Mountain spotted fever (Adams and Walker, 1981; Walker et al., 1978) and is compatible with the proposed mechanism of injury by the phospholipase-associated penetration mechanism. Further experiments should be designed to explore this and other direct rickettsial mechanisms of cytotoxicity other than soluble exotoxins and enzymes.

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INFECTION OF GENETICALLY IMMUNODEFICIENT MICE WITH *RICKETTIA CONORII*

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Summary. — In order to determine the definitive importance of T- and B-lymphocytes in immunity to *Rickettsia conorii*, mice genetically deficient in T-cells, B-cells, or both T- and B-cells were infected experimentally. T-lymphocytes rather than humoral antibodies were crucial to rickettsial clearance and a reduced mortality rate. Mice incapable of an antibody response to polysaccharide capsular antigens effectively controlled rickettsial infection with no mortality. In contrast, nude mice produced antibody to thymus-independent antigens early in the course of infection, yet experienced severe rickettsial infection resulting in deaths. The observed hepatic lesions are similar to those of boutonneuse fever. This model offers the opportunity to investigate rickettsial immune mechanisms and hepatic injury.

Key words: T-lymphocytes; B-lymphocytes; Rickettsia conorii; boutonneuse fever; liver

Introduction

The pathologic lesions of the *tache noire* and rash of boutonneuse fever and the inoculation site in guinea pigs experimentally infected with *Rickettsia conorii* contain rickettsiae infecting the vascular endothelium and leukocytes of the immune and phagocytic systems (Montenegro *et al.*, 1983*a*; *b*). The roles of each of these elements (rickettsiae and host response) in the pathogenesis of tissue injury were not determined. Likewise, the relative contribution of thymus dependent (T) lymphocytes and B-lymphocytes to immune recovery from *R. conorii* infection has not been defined. Investigations by Kokorin and co-workers suggested that T-lymphocytes are important for immunity to *R. conorii* (Kokorin *et al.*, 1982). Inbred DBA/2 mice were immunosuppressed by cyclophosphamide treatment and infected with *R. conorii*; the animals were given spleen cells from immune and non-immune donors. They demonstrated that immune spleen cells conferred protection and suggested that the T-lymphocyte was crucial for the de-

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velopment of immunity against *R. conorii*. This conclusion was based on the loss of protection after pretreatment of transferred immune spleen cells with antibodies inactivating T-cells.

Our work was designed to examine further the relative importance of T-and B-lymphocytes in the pathogenesis and immunity to *R. conorii*. We used mice genetically deficient in T-cells, B-cells as well as in both T- and B-cells, inoculated by intradermal route to examine the presence of rickettsiae and pathologic lesions at the inoculation site and in systemic tissues.

Materials and Methods

Animals. Thirty mice (generously provided by Dr. Don Mickey, University of North Carolina at Chapel Hill), included six 46-day-old male B-lymphocyte deficient (NIH II) mice (Group I) (Amsbaugh *et al.*, 1972), six 46-day-old male T-lymphocyte deficient (NIH nude) mice (Group II), six 46-day-old T- and B-lymphocyte deficient (NIH II) mice (Group III) (Azar *et al.*, 1980) and twelve 62-day-old male Swiss outbred immunologically intact mice (Group IV). Each group was housed in a separate cage except Group IV which was divided between two cages.

Rickettsiae. Rickettsia conorii (strain 7) was obtained from ATCC as a frozen 40% yolk sac suspension. Stock *R. conorii* was prepared by inoculation of the yolk sac of 5-day-old specific pathogen-free embryonated hen's eggs and harvested 3 days later. Aliquots were stored frozen at −70 °C as a 10% suspension in sucrose phosphate buffer. These aliquots contained 6×10^4 PFU/ml when titrated in primary chick embryo cell (CEC) culture.

Serology. Serum was evaluated for antibodies to *R. conorii* by indirect immunofluorescent antibody assay (Philip *et al.*, 1976) using microdots of *R. conorii* (strain 7) cultivated in rabbit kidney-13 (RK-13) cells. Slides with microdots were air-dried, fixed in acetone for 10 min, and stored frozen at −70 °C. Serial dilutions of mouse sera were incubated on microdots for 30 min at room temperature in a moist chamber. Slides were washed in phosphate buffered saline (PBS) for 30 min, reacted with fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulin (DAKO, Accurate Chemical and Scientific Company, Westbury, N.Y.), diluted 1 : 20, washed in PBS again, and mounted in 90% glycerol in PBS. Slides were examined on a Leitz Ortholux ultraviolet microscope with incident beam illumination equipped with barrier and exciter filters for FITC.

Histology and immunohistochemistry for spotted fever group rickettsiae. At the time of death, the inoculation site, spleen, and liver were collected, fixed in 4% neutral buffered formaldehyde for 24 hr, embedded into paraffin, and cut at 4 μm. Sections were stained with hematoxylin-eosin and by the Brown-Hopps method. Other sections were examined for *R. conorii* by immunofluorescence using a direct conjugate for spotted fever group rickettsiae (Hebert *et al.*, 1980) after deparaffinization and trypsin digestion (Walker and Cain, 1978; Montenegro *et al.*, 1983*a*, *b*).

Experimental design. Five animals from each type of immunodeficient mice and ten intact Swiss mice were inoculated with 0.2 ml of suspension containing 1.2×10^4 PFU of *R. conorii*. One animal in each of the six cages was marked for identification and inoculated with 0.2 ml of medium containing no rickettsiae. On day 3 one infected animal from each group was sacrificed by collecting blood from the heart under sodium-pentobarbital anesthesia. On day 7 two infected animals and one control animal from each group and on day 15 the remainder of the animals were similarly exsanguinated. Serum was separated from clotted blood. Serum specimens from the same animal group collected on the same day were pooled.

Results

Mortality due to *R. conorii* infection was observed only in T-cell deficient mice (2 deaths) and T- and B-cell deficient mice (1 death). The content of rickettsiae in tissues also followed the same pattern with more *R. conorii* in the livers and spleens of the T-cell deficient mice and T- and B-cell deficient mice than in the B-cell deficient mice and normal mice (Table 1). The humoral

Table 1. Distribution of Rickettsia conorii in the tissues of mice[a]

Animals	Day 3		Day 7		Day 15	
	Liver[b]	Spleen	Liver	Spleen	Liver	Spleen
B-deficient	0	0	+[b]	0.5[c]	0	0
T-deficient	0	0	+ + +	31	0	0
T- and B-deficient	0	0	+ + +	26	0	<1
Normal	0	0	+	2.5	0	0

a — All skin samples contained *R. conorii* on days 3 and 7. None had rickettsiae detected on day 15.
b — 0, no rickettsiae; +, few rickettsiae identified; + + +, many rickettsiae identified.
c — Number of *R. conorii*/ magnification field 40X.

immune responses to antigens of *R. conorii* are shown in Table 2. The absence of antibodies to *R. conorii* in B-deficient animals on day 7 and their delayed appearance on day 15 is consistent with a deficiency but not total absence of functional B-lymphocytes. The brisk production of antibodies by the T-cell deficient animals is presumably directed against T-independent carbohydrate-containing antigens. The mice that were deficient in both T- and B-lymphocytes were incapable of producing any antibodies.

Table 2. Antibody responses in mice infected with Rickettsia conorii[a]

Animals	Day 7	Day 15
B-deficient	0	+ +
T-deficient	+ +	+
T- and B-deficient	0	0
Normal	+	+ +
Control	N.D.	0

a — 0, <1 : 16; +, 1 : 16; + +, 1 : 128—1 : 1,024; N.D., not done

Hematogenous spread of rickettsiae to liver resulted in multifocal hepatocellular necrosis and surrounding inflammatory lesions in all groups of mice. These lesions comprised lobular foci of polymorphonuclear leukocytes and macrophages on day 3. By day 7 they had increased in number and assumed a granuloma-like appearance. Hepatocellular necrosis and admixture of polymorphonuclear leukocytes were more prominent in the T-deficient and B- and T-deficient mice (Fig. 1). These granuloma-like lesions persisted althogh less numerous in all animals on day 15. The lesions at the inoculation site were similar in all rickettsia-inoculated animals.

Discussion

Presented experiments in genetically immunodeficient mice document the importance of T-lymphocytes in the clearance of *R. conorii* from the tissues of infected mice. On day 7 the spleens and livers of T-cell deficient as well as of T- and B-cell deficient mice contained numerous rickettsiae, whereas animals with an intact T-lymphocyte immune system contained very few rickettsiae in their hepatic and splenic tissues. The only animals that died as a result of *R. conorii* infection in these studies were those deficient in T-lymphocytes whether or not they had B-lymphocytes. These results support the conclusion of Kokorin *et al.* (1982) that T-lymphocytes confer protection against *R. conorii* infection in mice. Our results, however, do not rely upon general immunosuppression after cyclophosphamide treatment, a drug which affects many tissues including not only T- and B-lymphocytes but also proliferating cells of the bone marrow and other tissues, and reconstitution with a mixture of mouse spleen cells that were either untreated or depleted of lymphocytes by an absorbed polyclonal antiserum principally designed to affect T-lymphocytes. Instead, these studies employed genetically immunodeficient mice that have deficiencies of specific components of the immune system.

Moreover, the generation of a humoral immune response in our experiments did not correlate with clearance of rickettsiae and protection from death. T-lymphocyte deficient nude mice synthesized antibodies to *R. conorii* detectable on day 7 at which time the visceral organs contained many rickettsiae. Despite the presence of antibodies in these animals, mortality was observed. Nevertheless, B-lymphocyte deficient mice had no antibodies and yet had effectively restricted rickettsial proliferation in the liver and spleen on day 7; moreover, none of these mice died. These observations conform to the conclusions of previous studies on immunity to members of the genus *Rickettsia*, namely that T-lymphocytes are crucial for immunity (Shirai *et al.*, 1976; Walker and Henderson, 1978; Murphy *et al.*, 1979; Kenyon and Pedersen, 1980; Kokorin *et al.*, 1982).

It is important to note that this does not exclude an auxiliary role for antibodies in opsonizing rickettsiae for phagocytosis and digestion particularly by macrophages (Gambrill and Wisseman, 1973; Beaman and Wisseman, 1976) or for specific antibodies to particular rickettsial proteins, e.g. a protein for attachment to host cells prior to entry into the cell, in blocking rickettsial penetration or another rickettsial function of pathogenic mechanism. Jerrells and Eisemann (1983) have suggested that the antibodies produced by nude mice to spotted fever group rickettsiae may be directed to the polysaccharide-containing slime layer described by Silverman *et al.* (1978). Nevertheless, this strain of B-deficient mice that has been shown to be defective in the production of antibodies to pneumococcal capsular polysaccharide (Amsbaugh *et al.*, 1972) effectively clears its tissues of *R. conorii*. Protection of guinea pigs against *R. rickettsii* by specific antibodies to a surface protein has been shown by Anacker *et al.* (1983) and by monoclonal antibodies to surface antigens to *Rickettsia rickettsii* (Lange, 1983, personal communication).

However, the natural generation of antibodies after initiation of infection does not seem to be as effective as incubation of rickettsiae with antibodies prior to inoculation (Anacker *et al.*, 1983) or prophylactic administration of antibodies (Ricketts and Gomez, 1908; Topping, 1940).

The mechanism by which T-lymphocytes confer immunity against rickettsiae has been suggested by recent in vitro studies of lymphokines (Nacy and Meltzer, 1979; Nacy and Osterman, 1979; Meltzer and Nacy, 1980; Meltzer *et al.*, 1982; Turco and Winkler, 1983*a*, *b*, *c*; Wisseman and Waddell, 1983). Lymphokines, γ-interferon in particular, exert rickettsiostatic effects on intracellular organisms not only in macrophages but also in fibroblasts and endothelial cells. Further evaluation of the importance of γ-interferon in vivo supports this as an important mechanism by which T-lymphocytes serve as a crucial host defense against rickettsiae (Palmer *et al.*, 1984).

The immune response has not been shown in this or previous studies to be a pathogenic mechanism of tissue injury in infections by members of the genus *Rickettsia* (Moe *et al.*, 1976; Mosher *et al.*, 1977; Walker and Henderson, 1978; Kenyon and Pederson, 1980; Kokorin *et al.*, 1982). The cytolytic effect of lymphokines on rickettsia-infected cells in vitro again raises the question of an immunopathologic mechanism of tissue and cellular injury in rickettsial diseases (Turco and Winkler, 1983*a*, *b*; Wisseman and Waddell, 1983). Although this investigation does not exclude a contribution to cell injury by lymphokine-mediated cytolysis, it does document that in the overall balance the T-lymphocyte affords protection against *R. conorii*. Other studies including the in vitro plaque model have demonstrated that rickettsiae possess direct cytopathic activity that appears to be mediated at least in part by the phospholipase-associated penetration mechanism (Walker and Cain, 1980; Winkler and Miller, 1982; Walker *et al.*, 1983) and is active in the absence of the immune system.

The similarity of the hepatic lesions of these animals to hepatic lesions in boutonneuse fever contributes to understanding of the pathogenesis of the human lesions of multifocal hepatocellular necrosis and associated focal hepatic inflammatory response (Guardia *et al.*, 1974; Faure *et al.*, 1977; Walker, Staiti, and Mansueto, unpublished data). The finding of *R. conorii* in these lesions in mice suggests that the rickettsiae play an important role in their pathogenesis. The immunodeficient mouse model of *R. conorii* infection should be useful in further dissection of pathogenic mechanisms of hepatic injury by *R. conorii* and in evaluation of the importance of stimulation of each component of the immune system by specific purified rickettsial antigens in protective immunity and vaccine design.

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Explanation to Micrographs (Plate LXIX):

Fig. 1. Clusters of immunofluorescent *Rickettsia conorii* (arrows) in a T-cell deficient mouse, (a) spleen (left) and (b) liver granuloma (right) 7 days after inoculation. Anti-spotted fever group rickettsiae FITC-labelled conjugate, ×375; c) Hepatic cell necrosis surrounded by nodular accumulation of polymorphonuclear leukocytes and macrophages in a T-cell deficient mouse 7 days after inoculation with *Rickettsia conorii*. Hematoxylin-eosin stain; ×375. Bar represents 10 μm.

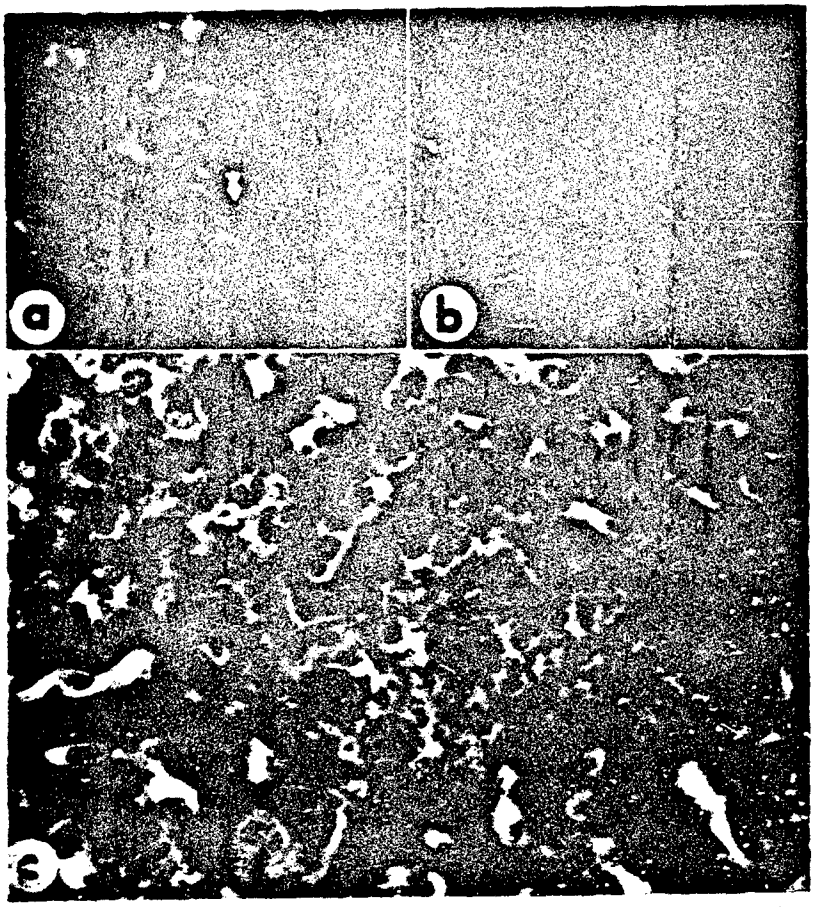

Fig. 1.

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CORRELATION OF THE DISTRIBUTION OF *RICKETTSIA CONORII*, MICROSCOPIC LESIONS, AND CLINICAL FEATURES IN SOUTH AFRICAN TICK BITE FEVER

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Abstract. Three South African patients with severe *Rickettsia conorii* infection had complicated courses of illness with 2 fatal cases and 1 with gangrene of multiple digits. Immunofluorescent organisms of *R. conorii* were demonstrated in vascular endothelium of brain, leptomeninges, renal glomerular arterioles and capillaries, renal arteries and veins, myocardial capillaries and arteries, pulmonary alveolar capillaries, pancreatic septa, splenic arterioles, and dermis. Rickettsiae were also observed in hepatic sinusoidal lining cells, splenic and lymph node macrophages, and the blood vessels of the partially viable zone of the amputated digits. Pathologic lesions included cerebral and cerebellar perivascular mononuclear leukocytes, mild mononuclear leptomeningitis, glomerular arteriolitis, vascular and perivascular mononuclear cell-rich inflammatory foci in the kidney, pancreas, skin, and myocardium, hepatocellular necrosis, and pulmonary edema. The sites of lesions and rickettsiae showed strong topographical correlation. Thrombi and hemorrhage occurred in a minority of the sites of vascular injury. Rickettsiae were the apparent direct cause of meningoencephalitis, peripheral gangrene, and other foci of vascular injury. Fatal *R. conorii* infection with disseminated organ involvement emphasizes the pathogenic potential of this disease.

Rickettsia conorii, a member of the spotted fever group of rickettsiae, is distributed widely through southern Europe, Africa, and the Middle East. Strains of *R. conorii* from this broad geographic distribution have been assigned to this species on the basis of quantitative measurements of the capability of antisera to neutralize the lethal effect of intravenous inoculations of the respective rickettsiae.[1] The disease caused by *R. conorii* has borne various names reflecting the geographic occurrence including Mediterranean spotted fever, Kenya tick typhus, South African tick bite fever, and India tick typhus. The major ecologic niche of the rickettsiae affecting man is the dog tick, *Rhipicephalus sanguineus* which maintains the rickettsia by transovarial transmission and transmits the rickettsia to man by its bite. Other ticks which have been reported to harbor *R. conorii* include members of the genera *Rhipicephalus, Ixodes, Dermacentor, Hyalomma, Amblyomma, and Hemaphysalis*. It is possible that some rickettsial genetic diversity may exist among these geographic and ecologic sources. Although human infection with *R. conorii* is generally considered to be a self-limited illness of moderate severity, the range of clinical severity contains subjects with antibodies to *R. conorii* and no history of febrile exanthem,[2,3] a patient with *tache noire* and no other signs or symptoms,[4] patients with severe manifestations such as acute renal failure, neurologic signs, or peripheral gangrene,[5–7] and fatal cases.[6–8] Severe cases have been observed in Israel, South Africa, France, and Italy. Host factors which may affect the severity of illness are glucose-6-phosphate dehydrogenase deficiency[9] and older age.[6–8] Specific rickettsial virulence factors have not yet been identified. The pathologic alterations associated with human *R. conorii* infection have been reported only in skin and liver.[10–13, 15, 16] We describe the pathology and rickettsial distribution in the central nervous system, kidney, liver, heart, lung, spleen, lymph node, pancreas, adrenal, and skin. These are the first descriptions of the pathology and rickettsiae in the tissues of fatal *R. conorii* infection.

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MATERIALS AND METHODS

Paraffin sections of tissues from 3 cases of South African tick bite fever were stained by routine hematoxylin-eosin method, phosphotungstic acid-hematoxylin technique, and deparaffinization-trypsin digestion-immunofluorescence method with a conjugate reactive with *R. conorii* as reported in detail previously.[12,17] Tissues examined were cerebrum, cerebellum, kidney, liver, spleen, heart, lung, lymph node, and adrenal gland (Case 1); cerebrum, kidney, liver, spleen, heart, lung, and pancreas (Case 2); and gangrenous digits (Case 3). Positive control tissues for *R. conorii* immunofluorescence included eschar from guinea pig inoculated intradermally with *R. conorii* (strain 7), eschars from patients with serologically documented boutonneuse fever, and liver tissue from nude mouse inoculated with *R. conorii* (strain 7).

RESULTS

Clinical observations

Case 1. A 54-year-old miner employed on one of the gold mines of the Witwatersrand became ill with "flu-like" symptoms of headache, muscle pain and fever. He became worse on each succeeding day and on the fourth day developed a profuse rash and was admitted to the hospital. On the ninth day, the rash became hemorrhagic, and he was transfered to the Rietfontein Fever Hospital near Johannesburg.

On admission he was desperately ill, being semicomatose with stertorous breathing. An extensive hemorrhagic maculopapular rash covered his body. A nonpurulent ulcer was seen in the middle of his back. His tongue was coated and palate covered with blood-stained mucus. His chest sounds were obscured by stertorous breathing, heart rate was 100/min, and blood pressure, 140/80 mm Hg. He had slight neck and back stiffness. His deep tendon reflexes were brisk, and the Babinski sign was flexor. It was suspected that he had tick bite fever.

Because of renal failure and urgent need for dialysis, he was transfered to the intensive care unit in the Johannesburg Hospital, but died soon after admission.

Laboratory data included hemoglobin 15 g/dl, white blood cell count 10,500/μl with 86% neutrophils, many showing toxic granulation, 7% monocytes, and 7% lymphocytes, platelet count 65,000/μl, prothrombin index 68%, serum sodium 121 mEq/l, serum potassium 4.9 mEq/l, blood glucose 22 mg/dl, blood urea 42 mmol/l, and creatinine 415 μmol/l. His urine contained numerous red blood cells and white blood cells, protein, and glucose. His cerebrospinal fluid was slightly xanthochromic and contained 1,280 red blood cells, 3 neutrophils, 11 lymphocytes, protein 93 mg/dl, and glucose 11.2 mmol/l. No bacteria were observed and bacterial culture yielded no growth. Virus was not isolated from his blood by the inoculation of litters of baby mice and tissue cultures. Two adult male guinea pigs inoculated with his blood on the day of admission to Rietfontein Hospital developed a slight fever associated with a slight scrotal reaction. No passages were made. When their sera were tested a month later in the rickettsial complement fixation test, a positive reaction with *Rickettsia conorii* var. *pijperi* antigen was noted, thus confirming the clinical diagnosis of South African tick bite fever.

It was subsequently ascertained that his family owned a dog which was allowed indoors and to sleep on the patient's bed. It was dipped regularly to rid it of the ticks which it acquired in the vicinity of the house. The patient had complained of a "pimple" in the middle of his back 4 or 5 days before he became ill. A plaster was put on it and when it was removed left a nonpurulent sore. It seems likely that this was the primary lesion of tick bite fever and that the tick responsible for transmitting the infection came from the dog sleeping on his bed.

Case 2. A 65-year-old man, after returning to his home in Hillcrest, Durban, from a holiday in the inland farming district of Mooi River, became ill with high fever and severe headache. He became delirious and developed a profuse maculopapular rash which became hemorrhagic. Because of his rash and other signs of a hemorrhagic state, the possibility of one of the African hemorrhagic fevers was considered. In spite of all treatment, he died about 14 days after the onset of his illness. At postmortem, specimens were taken from the varous organs and tissues. Serum specimens separated from blood taken on 18 and 25 August, the day before he died, were also evaluated. Indirect immunofluorescent tests for antibodies to Lassa, Marburg, Ebola, Crimean-Congo, and Rift Valley fever viruses gave negative results with both serum specimens. The

complement fixation tests for antibodies to rubeola and herpes simplex viruses were not diagnostic, but the complement fixation test demonstrated a rise in antibody titer to antigens of *R. conorii* var. *pijperi* from 1:8 on 18 August to 1:64 on 25 August.

Case 3. A 73-year-old English-born widow had rheumatic fever when 7 years old, but had no heart trouble after the attack. She had come to South Africa about 5 years prior to the onset of illness and lived in a suburb north of Johannesburg. She had 2 dogs which were restricted to her garden but were allowed indoors. In May 1980 she was admitted to the hospital for a chest infection which responded well to antibiotic treatment. At that time she was found to have mitral stenosis and regurgitation and subsequently experienced mild symptoms of shortness of breath and palpitations.

About a week before admission to the hospital, she felt tired and complained of muscle and joint pains. Her daughter noted that she had been feverish and delirious for 2 nights. After developing a profuse rash, she was admitted to the Johannesburg Hospital with a provisional diagnosis of septicemia.

At admission on 5 October 1980, she was somewhat confused. Her temperature was 39°C, pulse rate (at times irregular) was 120/min, and blood pressure, 94/50 mm Hg. She had an extensive maculopapular rash on a background of cyanotic suffusion of the skin with scattered petechiae and ecchymoses, especially marked peripherally. The capillary fragility test was positive. She had slight neck stiffness and muscle tenderness. There was slight cardiomegaly, a short systolic and a mid-diastolic murmur and atrial fibrillation. Her liver was slightly enlarged and tender, but her spleen was not palpable. Infective endocarditis was suspected, and she was treated with large doses of antibiotics. Laboratory data incuded 5 negative blood cultures taken before the institution of antibiotic treatment, presence of fibrin degradation products in plasma gross hematuria with many red cell casts, and blood creatinine 520 μmol/l. Her condition continued to deteriorate, and she became stuporous and then lapsed into a coma. On the fourth day of hospitalization, the clinical findings were reviewed. The diagnosis was changed to tick bite fever, and treatment with 500 mg tetracycline every 6 hr was initiated. The patient defervesced during the next 3 days, but her condition remained grave. She developed gangrene of all her finger tips except for the left small finger and thumb and of her second and third toes of her left foot. On 13 November the gangrenous tissue was amputated. The postoperative course was uneventful, and she was discharged relatively well. The Weil Felix test revealed seroconversion by *Proteus* OX19 agglutination, thus suggesting the diagnosis of a rickettsial disease.

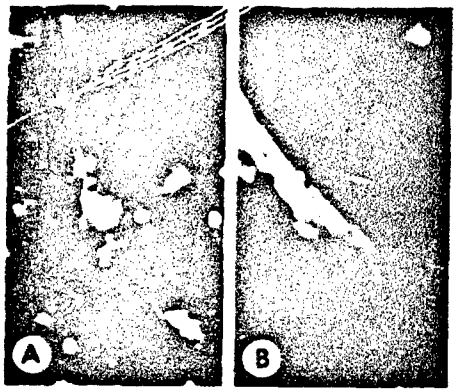

FIGURE 1. Photomicrographs of immunofluorescent *R. conorii* demonstrated by anti-spotted fever group rickettsial conjugate in fatal South African tick bite fever. A. Rickettsiae in endothelial location of a renal blood vessel in Case 2, ×375. B. Rickettsiae in the cytoplasm of a macrophage in the marginal sinus of a lymph node in Case 1, ×380.

Immunofluorescence observations

Thin, bright green immunofluorescent organisms were observed only in the locations of vascular endothelium in many sites (Fig. 1A) and of macrophages in hepatic and splenic sinusoids and lymph node sinuses (Fig. 1B). Nonspecific staining of host tissues was not seen. In Case 1 the brain contained numerous rickettsiae in cerebral and cerebellar blood vessels and foci of rickettsiae in the subarachnoid location of the leptomeninges. Rickettsiae were observed in glomerular arterioles and capillary tufts, intertubular blood vessels, and arterial endothelium. In the liver, rickettsiae were identified only in a few sinusoidal lining cells; no rickettsiae were seen in hepatocytes. Splenic rickettsiae appeared to be in macrophages and arteriolar endothelium. Few foci of *R. conorii* were observed in capillaries between myocardial cells, pulmonary al-

FIGURE 2. Photomicrographs of brain from fatal South African tick bite fever (Case 2) showing perivascular infiltration of the neuropil by mononuclear leukocytes, hematoxylin-eosin stain, ×240. Inset, Immunofluorescent *R. conorii* (originally bright green) in a cerebral blood vessel from the same patient. Autofluorescence in the neuropil represents neuronal lipofuscin (originally orange), anti-SFG rickettsial conjugate, ×375.

FIGURE 3. Photomicrograph of renal glomerulus in Case 2 with numerous immunofluorescent *R. conorii* in the walls of the glomerular arteriole and capillary tufts. Anti-SFG rickettsial conjugate, ×375.

veolar septa, and macrophages within marginal and draining sinuses of a lymph node. No rickettsiae were demonstrated in adrenal gland but were present in blood vessels in periadrenal adipose and connective tissue.

In Case 2, numerous rickettsiae infected cerebral blood vessels (Fig. 2, inset), glomerular arterioles and capillary tufts (Fig. 3), renal arterial endothelium, and intertubular blood vessels near the corticomedullary junction (Fig. 4). Few hepatic rickettsiae were observed in scattered sinusoidal lining cells. In spleen, rickettsiae were identified in arterial endothelium and macrophages in small quantities and in one medium-sized artery in a large amount. In the heart, a few foci of rickettsiae were present in arterial endothelium and capillaries between myocardial fibers. Very few rickettsiae infected pulmonary alveolar capillaries. Small foci of rickettsiae were seen in capillaries and septal blood vessels of the pancreas and blood vessels in the dermis of skin.

In Case 3, several foci containing numerous *R. conorii* were identified in the injured, partially necrotic blood vessels at the margin between viable and necrotic tissue (Fig. 5). Rickettsiae were not observed in the mummified necrotic zone or in the zone of healthy tissue.

Microscopic lesions

In Case 1, the cerebrum and cerebellum contained numerous foci of perivascular mononuclear cells some of which infiltrated the adjacent neuropil. There was widespread enlargement of

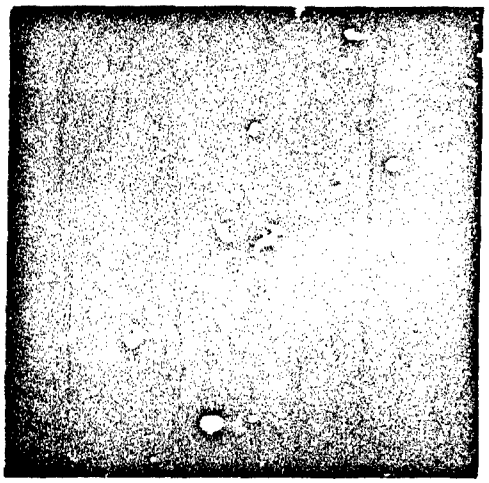

FIGURE 4. Photomicrograph of numerous immunofluorescent *R. conorii* in a focus of vasculitis and perivasculitis near the corticomedullary junction of the kidney in Case 2. Anti-SFG rickettsial conjugate, ×235.

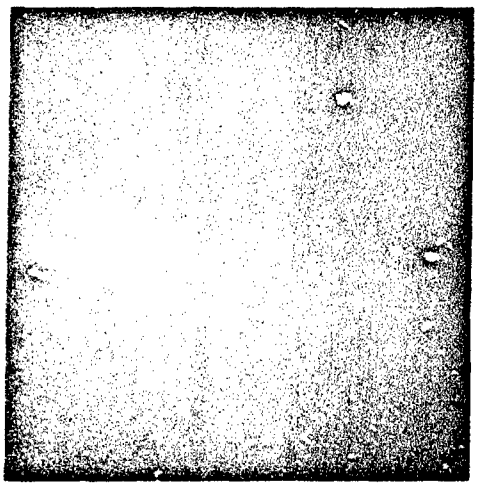

FIGURE 5. Photomicrograph of numerous *R. conorii* in a blood vessel of the partially viable zone of an amputated gangrenous finger (Case 3). The blood vessel is narrowed by a nonocclusive thrombus. Anti-SFG rickettsial conjugate, ×235.

endothelial cells and a diffuse mild increase in microglial cells infiltrating the neuropil. The subarachnoid space had a mild hemorrhage with erythrophagocytosis by macrophages. The kidney was severely autolytic, but multiple foci of mononuclear leukocytic vasculitis could be identified in the outer part of the medulla near the corticomedullary junction. Hepatic lesions included multifocal, randomly distributed coagulative necrosis of solitary hepatocytes (Fig. 6), few polymorphonuclear leukocytes and moderate quantities of small lymphocytes in portal triads, mild steatosis, moderate congestion, and mild sinusoidal leukocytosis. There were no hepatic granulomas, portal vasculitis, or leukocytic accumulation around necrotic hepatocytes. Matching of serial sections by bright-field microscopy and immunofluorescence demonstrated necrotic hepatocytes adjacent to infected sinusoidal lining cells and *R. conorii* within splenic arterioles that contained thrombi and karyorrhectic debris. The red pulp was congested, and no germinal centers were observed. The myocardium contained a few foci of interstitial leukocytes, predominantly mononuclear cells. Lung lesions included protein-rich pulmonary edema, congestion, focal nonocclusive thrombosis, focal acute pneumonia with alveolar polymorphonuclear leukocytic exudate, and foci of fibrosis containing carbon pigment and birefringent silica crystals. Lymph nodes also contained anthracosilicotic fibrosis. There were foci of adrenocortical necrosis with surrounding leukocytic response, and two periadrenal arteries had foci of acute vascular wall necrosis (Fig. 7).

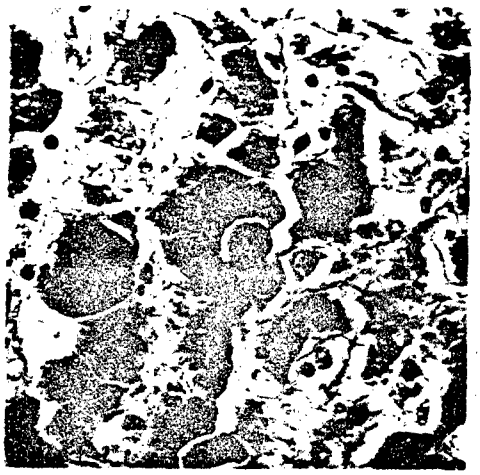

FIGURE 6. Photomicrograph of liver (Case 1) shows an example of a rounded, shrunken necrotic hepatocyte (Councilman-like body). Hematoxylin-eosin stain, ×380.

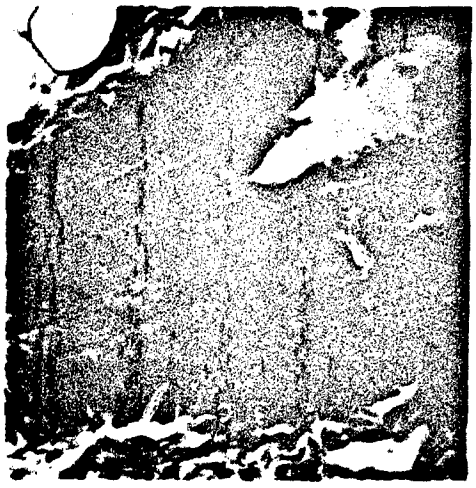

FIGURE 7. Photomicrograph of an artery in periadrenal adipose tissue of Case 1. This oblique section through the arterial wall demonstrates marked karyorrhexis and inflammation limited to the endothelium and subendothelial intima and sparing the media. Hematoxylin-eosin stain, ×240.

FIGURE 8. Photomicrograph of kidney from Case 2 shows necrotizing glomerular arteriol tis. Hematoxylin-eosin stain, ×240.

FIGURE 9. Photomicrograph of kidney from Case 2 demonstrates a focus of severe vasculitis and perivasculitis in the outer part of the medulla (focal perivascular interstitial nephritis). Hematoxylin-eosin stain, ×150.

In Case 2, many foci of vasculitis in the brain consisted of mononuclear leukocytes infiltrating the blood vessel wall and the surrounding neuropil (Fig. 2). There was also a mild mononuclear leukocytic leptomeningitis. Lesions in the severely injured kidney included karyorrhexis, thrombosis, and leukocytic infiltration of glomerular arterioles (Fig. 8), karyorrhexis in glomerular capillary tufts, cortical vasculitis with perivascular mononuclear leukocytes, plasma cells, and focal hemorrhage, multifocal cortical interstitial edema, and multifocal severe vasculitis (Fig. 9) at the corticomedullary junction and in the outer part of the medulla. The liver contained multifocal necrosis of solitary hepatocytes, intracanalicular cholestasis, mild steatosis, and mitosis. No hepatic granulomas, portal vasculitis, or leukocytic response to necrotic hepatocytes were observed. The spleen had a capsular

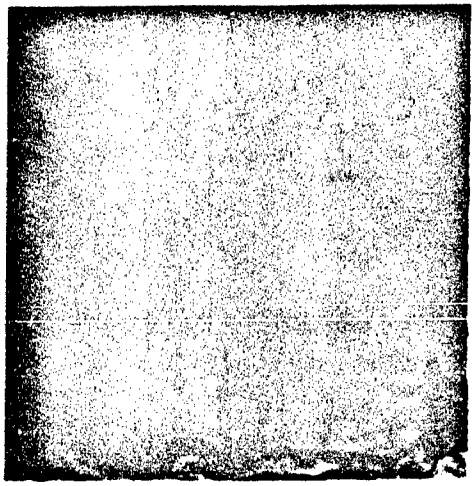

FIGURE 10. Photomicrograph of skin from Case 2 shows severe vascular injury with a nonocclusive luminal thrombus. Hematoxylin-eosin stain, ×240.

hemorrhage and polymorphonuclear leukocytes and plasma cells in the red pulp. The lungs were congested with intraalveolar amorphous eosinophilic material compatible with pulmonary edema, scattered intraalveolar erythrocytes, and deposits of carbon pigment. The pancreas was autolyzed, but contained a focus of identifiable perivascular mononuclear cell infiltrate. The skin showed multifocal dermal and subcutaneous vasculitis with perivascular edema and leukocytes including polymorphonuclear leukocytes. Two dermal blood vessels contained thrombi that did not occlude the lumina (Fig. 10).

In Case 3, the amputated fingers had 3 zones: mummified necrotic tissue, a viable margin at the line of resection, and an intervening partially viable zone. The partially viable zone contained thrombosed blood vessels which consisted of a mixture of viable and necrotic cells (Fig. 11A). The tissue at the line of resection was a healthy band of granulation tissue containing recanalized thrombosed vessels with a remnant of mononuclear inflammatory cells (Fig. 11B). Few morphologic details were discernible in the mummified necrotic area.

DISCUSSION

The three cases of South African tick bite fever illustrate many of the diagnostic and clinical problems of infection with ixodid tick-transmitted *R. conorii* var. *pijperi*. The diagnosis of tick bite fever is made provisionally by epidemio-

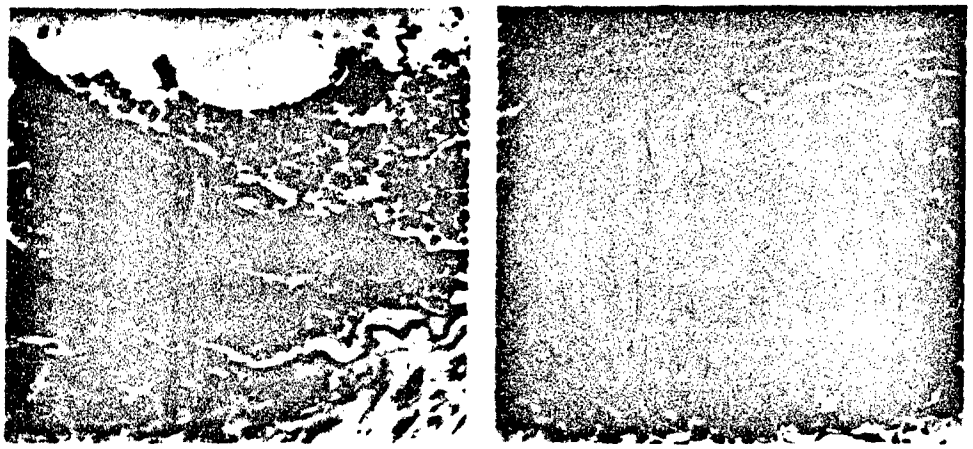

FIGURE 11. Photomicrographs of tissue from the amputated gangrenous fingers in Case 3. Both hematoxylin-eosin stain, ×210. A. A nonocclusive luminal thrombus contains several morphologically intact cells although cells of the vessel wall are necrotic as determined by nucleolysis. This focus contained immunofluorescent *R. conorii*. B. A healing blood vessel with a recanalized luminal thrombus, fibroblasts, and remnants of mononuclear inflammation in the excised margin of viable tissue. This region contained no *R. conorii* organisms.

logic and clinical observations, treatment with tetracycline is administered, and subsequently the diagnosis is confirmed by laboratory methods. The patients often give a history of having been camping or picknicking in the bushveld, where ticks abound, one week before becoming ill. Clinically the diagnosis is suggested by the presentation of a primary sore with a black necrotic center, associated especially in severe cases with tender regional lymphadenitis, fever, and the development of a maculopapular rash on the third to fifth day of illness. The diagnosis can be confirmed in the laboratory by serology, by the isolation of the causative rickettsiae, and by immunofluorescent demonstration of spotted fever rickettsiae in a biopsy of the eschar or rash.[12-14] Reliance is usually placed on the results of the serological tests; however, these tests usually do not give positive results until after the tenth day of illness. Isolation and characterization of *R. conorii* by intraperitoneal inoculation of guinea pigs with the patient's blood is performed only in specialized laboratories. As the specific immunofluorescent demonstration of spotted fever rickettsiae in skin biopsies is only available in a few laboratories, the early diagnosis in most cases of tick bite fever must be based on the clinical findings. Treatment with tetracycline or chloramphenicol should be initiated immediately to avoid the pathologic consequences illustrated in these patients.

In a typical case, the clinical course is characterized by complaint first of lassitude most marked in the evening. A feeling of chillness with slight rigors may then occur, and the patient develops fever and headache, which may become so severe as to be almost unbearable. The patient may complain of insomnia and become delirious, most often at night. The temperature falls on about the tenth day. In children and young adults, there are few complications and recovery is rapid. In the absence of treatment, complications are frequent in older patients and may occur even with treatment.

The pathologic lesions and pathophysiology of fatal Rocky Mountain spotted fever (RMSF) and South African tick bite fever have both similarities and distinct differences. In general, the pattern of mononuclear leukocyte-rich infiltration of the infected blood vessel wall and perivascular space is the basic lesion in most affected organs in both RMSF and these two fatal cases of *R. conorii* infection. Severely ill patients with either disease may develop a hemorrhagic state with bleeding into the elements of the rash, petechial hemorrhage and purpura of the skin, and bleeding from needle puncture wounds and from the mucous membranes. The hemorrhagic state may be associated with a marked thrombocytopenia, prolonged prothrombin time, and evidence of a consumption coagulopathy. The lesions in the central nervous system from both diseases resemble the classic Frankel's nodules of typhus fever with cells compatible with macrophages and small and large lymphocytes permeating the perivascular neuropil.[18, 19] A mild leptomeningitis is observed in both rickettsioses. The cerebrospinal fluid in Case 1 reflected the rickettsial meningoencephalitis with mild pleocytosis and elevated protein concentration. Clinically, there may also be marked involvement of the central nervous system in both South African tick bite fever and RMSF, progressing from severe headache to mental confusion, delirium and coma. Other analogous lesions include multifocal perivascular interstitial nephritis,[20, 21] focal interstitial myocarditis,[22, 23] dermal and subcutaneous vasculitis in the skin of the rash, eschar, and peripheral gangrene[10-13, 24, 25] and vasculitis in the pancreatic interlobular septa[26] and periadrenal adipose tissue. Lesions which were observed in fatal *R. conorii* infection and are not characteristic of RMSF are necrotizing glomerular anteriolitis[20, 25, 27] and multifocal necrosis of single hepatocytes. Although hepatocellular necrosis other than centrilobular necrosis was present in 5 of 9 fatal cases of RMSF[28] in one series and in 7 of 16 cases in another series (M. Jackson and W. D. Bradford, Duke University Medical Center, C. Kirkman and D. H. Walker, personal communications), the major hepatic lesions of RMSF, portal vasculitis and triaditis, were not observed in the two patients presented. Clinically, hepatic involvement is reflected in some cases of *R. conorii* infection by jaundice and increased levels of blood bilirubin and hepatic enzymes. The other major difference is the presence of interstitial pneumonia in RMSF[29, 30] and its absence in South African tick bite fever.

The distribution of lesions correlates with the locations of rickettsiae in each disease. Thus, the pulmonary capillaries and other small pulmonary blood vessels are infected with numerous *R. rickettsii* in RMSF, but there were few *R. conorii* infecting the pulmonary microcirculation.[29] Conversely, the glomerular arterioles in

South African tick bite fever were the sites of intense *R. conorii* infection and necrotizing vasculitis but are not a target in RMSF.[20, 21, 27] In both, renal failure may be reflected by rising blood urea and creatinine levels and ultimately anuria. The location of *R. conorii* and lesions correlated well in brain, meninges, liver, kidney, heart, spleen, skin, pancreas, and periadrenal arteries. These general observations were confirmed in specific foci where *R. conorii* were observed in serial sections of loci of vascular injury. Thus, these data support the theory of direct rickettsial injury of the parasitized cells proposed by Wolbach[24] which is also supported by recent in vitro experiments.[31–33] A discrepancy in the correspondence of the injured cell and parasitized cell is noted in the liver. The description of *R. conorii* infection of sinusoidal lining cells is the first report of direct rickettsial infection of the liver in this disease; nevertheless, necrosis occurs in the hepatocyte adjacent to the infected sinusoidal lining cell. The mechanism by which this injury to the hepatocyte is mediated is not apparent. Previous investigations of lesions in hepatic biopsies have demonstrated accumulations of leukocytes in foci of hepatocellular necrosis.[15, 16] We have been unable to demonstrate intact *R. conorii* in any of these inflammatory lesions in human liver biopsies. Experimental infections of mice with *R. conorii* have suggested that immune mechanisms clear the rickettsiae efficiently from the foci of hepatocellular necrosis and inflammation of mice with intact T-lymphocytes while these lesions in T-cell-deficient mice contain numerous rickettsiae.[34] Moreover, those experiments and the absence of lymphocytes and macrophages in the foci of hepatocellular necrosis at the stage of development present in these cases would make cell-mediated immunopathologic mechanisms seem unlikely. It may be hypothesized that the patchy hepatocellular necrosis is related to the infected adjacent sinusoidal lining which may cause reduced perfusion of the liver lobule and diminished exchange of nutrients and metabolites between the lumen of the sinusoid and the hepatocyte.

The other lesions of particular interest are in the amputated fingers. In addition to the expected observations of necrosis and wound repair, there was a zone of severely ischemic tissue in which a few viable cells persisted and organisms of *R. conorii* were identified. The observation of persistent rickettsiae in the tissues 36 days after the beginning of treatment with tetracycline is remarkable. Identical findings were present in amputated leg specimens from a patient in North Carolina with Rocky Mountain spotted fever who had been treated for three weeks with chloramphenicol (D. H. Walker, personal communication). In both cases the spotted fever group rickettsiae were seen only in the ischemic, partially viable zone where delivery of effective antirickettsial drug concentrations and host defenses such as T-lymphocytes, macrophages, and interferon was probably inadequate. This phenomenon may be related to the well-known capability of spotted fever group rickettsiae to continue to proliferate in the yolk sac of hen's eggs for 48–72 hr after the death of the embryo.[35] Proof that these morphologically intact organisms are truly viable will require isolation of rickettsiae from similar amputation specimens. This documentation of the pathologic potential of *R. conorii* in its most severe form indicates that further investigations of the renal, hepatic, neurologic, and cutaneous pathophysiology of this disease and of the pathogenic mechanisms of *R. conorii* should be pursued.

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DEMONSTRATION OF SPOTTED FEVER GROUP RICKETTSIAE IN THE TACHE NOIRE OF A HEALTHY PERSON IN SICILY*

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Abstract. A human case of rickettsial infection occurred in Sicily following tick bite. The patient did not have fever, the typical nodular rash, or other symptoms of illness other than development of a tache noire containing spotted fever group rickettsiae, which were demonstrated by immunofluorescence. A high titer of antibodies of the IgG class suggests that the patient may have had previous exposure to *Rickettsia conorii* or a related spotted fever group rickettsia. An anamnestic response may be hypothesized to have conferred partial immunity, with resulting containment of rickettsiae at the site of inoculation.

Boutonneuse fever (BF) is a tick-borne spotted fever group rickettsiosis caused by *Rickettsia conorii*, and is generally transmitted by the brown dog tick, *Rhipicephalus sanguineus*. In 50% of clinically apparent cases of BF, a lesion occurs at the site of the observed or presumed tick bite. BF is further characterized by fever, cutaneous nodules and occasionally involvement of visceral organs. The documentation of asymptomatic infection with *R. conorii* by serologic surveys of persons in whom there was no history of BF suggests the hypothesis that there may be a spectrum of pathogenicity of the host-parasite relationship between humans and *R. conorii*-like rickettsiae.[1] This spectrum may include asymptomatic seroconversion, tache noire without other signs or symptoms, and various combinations of tache noire, fever, rash, and cardiovascular, pulmonary, renal, and hepatic complications. This report documents for the first time the occurrence of a spotted fever group rickettsial infection with a tache noire but no other manifestations of disease.

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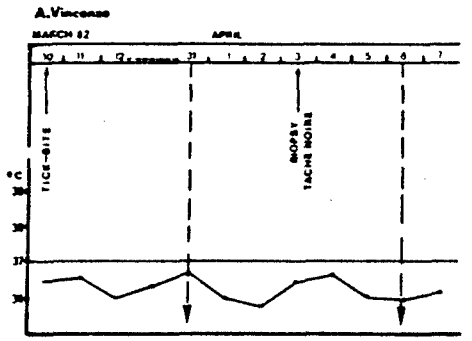
CASE REPORT

A 60-year-old agricultural laborer from Sciacca, Italy noted the presence of a tick on the lateral aspect of the right lower leg and removed it on 10 March 1982. Twenty days later he sought medical attention because of the development of a skin lesion at the site of the tick bite. The cutaneous lesion consisted of a central, black ulcer 2 cm in diameter surrounded by vesicles and a peripheral erythematous zone (Fig. 1). Data including clinical course, rickettsial serology, serum immunoglobulins and complement are shown in Figure 2. Indirect immunofluorescent antibody assay confirmed the diagnosis of BF with anti-*R. conorii* IgG titer of 1:320 and IgM titer of 1:80.

On 3 April 1982, a biopsy of the tache noire was performed. Bright field microscopy revealed foci of pseudoepitheliomatous hyperplasia in the epidermis surrounding the necrotic mass of karyorrhectic debris, fibrin and keratin, corresponding to the eschar (Fig. 3). In the surrounding dermis and underlying subcutaneous tissue, there were perivascular accumulations of macrophages, lymphocytes, and numerous eosinophils. The vascular endothelial cells were swollen. Examination of sections by the method of deparaffinization and trypsin digestion,[2] followed by direct immunofluorescence with a conjugate reactive with spotted fever group rickettsiae,

FIGURE 1. Tache noire in a patient without clinically apparent boutonneuse fever.



W-F	neg	neg
IF IgTot	640	1280
IgG	320	320
IgM	80	160
C3c	110	168
C4	61	85
IgA	92	304
IgG	240	240
IgM	48	48

FIGURE 2. Clinical course, rickettsial serology, serum immunoglobulins, and complement in a patient without clinically apparent boutonneuse fever. WF, Weil Felix; IF, immunofluorescence; C3c, C4, IgA, IgG and IgM (radial diffusion on Petri plates, Behring), normal values, respectively, 50–120; 20–50; 90–450; 800–1,800; 62–250 mg/100 ml.

showed focal clusters of coccobacillary organisms in the lining of the vessel walls in the reticular dermis (Fig. 4). The immunofluorescent conjugate was prepared at the Centers for Disease Control, using killed *R. rickettsii* as antigen for immunization of rabbits.[3] The conjugate of the globulin fraction of rabbit antiserum has also been demonstrated to react with *R. conorii* at a titer of 1:512.

Reaction of sections of the eschar with guinea pig preserum by indirect immunofluorescence using a 1:20 dilution of serum and 1:40 dilution of rabbit anti-guinea pig IgG conjugate (DAKO, Accurate Chemical and Scientific Corporation, Westbury, N.Y.) revealed no organisms, whereas the same indirect immunofluorescent system using convalescent serum collected from the same animal 1 month after inoculation of *R. conorii* revealed foci of thin bacilli compatible with rickettsiae.

On 5 April the anti-*R. conorii* titers remained at the same level while both the third and fourth components of complement were slightly elevated. At no time during his course did the patient report a fever. He was afebrile and did not have a rash during evaluation of the eschar or during the following 8 months.

DISCUSSION

The observation of spotted fever group rickettsiae at the site of tick bite is strong evidence for inoculation of rickettsiae into the skin by tick bite, colonization of vascular endothelium by the rickettsiae, and stimulation of host defenses without any systemic signs or symptoms of disease. In our patient rickettsiae were identified in the tache noire by direct immunofluorescence using rabbit anti-*R. rickettsii* fluorescein conjugate.[2] This conjugated antiserum also reacts with *R. conorii*, as shown by Hebert et al.,[3] and can be considered as specific for the spotted fever group of rickettsiae.

The fact that rickettsiae persisted for 23 days after tick bite is surprising, but has previously been documented to occur in rickettsial infections. *R. rickettsii* has been recovered from lymph nodes of a patient 1 year after clinical recovery from Rocky Mountain spotted fever.[4] Even in

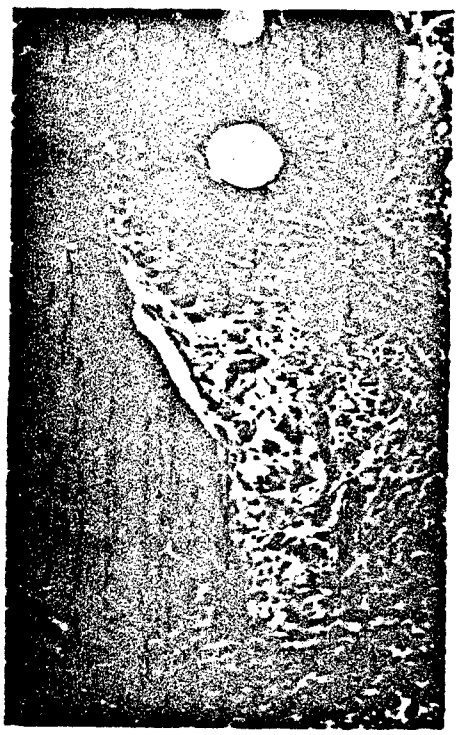


FIGURE 3. Photomicrograph of eschar with focal dermal necrosis (lower left), downgrowth of stratified squamous epithelium along tract of tick bite, adjacent granulation tissue, chronic inflammation, and swollen endothelial cells. Hematoxylin-eosin, ×235.

FIGURE 4. Biopsy of tache noire. Fluorescent rickettsiae in wall of dermal vessel. Fluorescein isothyocianate-conjugated anti-spotted fever group rickettsiae serum. ×300.

vitro in the plaque model, the center of the plaque has been shown to harbor a few intact cells containing rickettsiae after most of the surrounding cells have been destroyed.[5] These observations suggest that selection of less virulent rickettsiae or more resistant host cells may occur during infection, allowing for asymptomatic persistence of rickettsiae. Persistence of typhus rickettsiae in patients for many years after epidemic typhus may result in recrudescent illness. Our patient's tache noire was still in an active phase with early manifestations of healing 23 days after tick bite, whereas most of these lesions heal between 14 and 20 days. It is conceivable that this patient's humoral immune response prevented the spread of rickettsiae, while deficient cell mediated immunity failed to eliminate the intracellular organisms. Experiments with *R. mooseri* in guinea pigs support this hypothesis, since passive transfer of immune cells prevented an inoculation site lesion while passive transfer of immune serum failed to prevent the lesion.[6]

The two principal hypotheses that may explain the occurrence of *R. conorii* infection manifested only by an eschar are either that the strain of *R. conorii*-like rickettsia was of relatively low virulence, or that previous spotted fever group rickettsial infection provided partial immune protection. Investigation of spotted fever group rickettsiae in North America has revealed a great diversity of rickettsial species with a wide spectrum of virulence as judged by response of guinea pigs to inoculation.[7] At the present time the range of virulence of *R. conorii*, and possibly other spotted fever group rickettsiae in the Mediterranean basin, is not known. The serologic documentation of infection with *R. conorii* among

as many as 20% of persons in western Sicily who are engaged in agricultural activities and give no history of BF suggests that there are nonpathogenic strains of *R. conorii* in Sicily.[1,8] The possibility of a previous infection with *R. conorii* cannot be excluded. The serology, in fact, demonstrated a higher level of antibody to *R. conorii* in the IgG class than in the IgM class, as would be expected in an anamnestic immune response. Bourgeois et al.[9] have shown that in primary infection with *R. tsutsugamushi* the antibody response is mainly of the IgM class. In contrast, reinfection scrub typhus stimulates an antibody response mainly of the IgG class. We have also observed these two types of antibody response to *R. conorii* in BF.[10] It may be hypothesized that our patient, an agricultural laborer who is at high risk for both tick bite and *R. conorii* infection, had a prior infection with residual immunity sufficient to contain the subsequently inoculated organisms at the portal of entry. Elucidation of the host-rickettsia relationship between humans and *R. conorii* will require extensive investigation of strains of *R. conorii* isolates in Sicily, and of the immunology of human host defenses against rickettsiae. BF offers an excellent opportunity for the advancement of knowledge concerning rickettsial pathogenesis and immunity.

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Effect of Synthetic Protease Inhibitors of the Amidine Type on Cell Injury by *Rickettsia rickettsii*

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To evaluate the importance of proteolytic activity in the pathogenesis of cell injury by *Rickettsia rickettsii*, a series of four aromatic amidine inhibitors of trypsin-like proteases were introduced into the plaque model. The compounds were shown to be active toward plaque reduction with their order of effectiveness parallel to their antitrypsin activity. One of the compounds, bis(5-amidino-2-benzimidazolyl)-methane, at a concentration of 10^{-5} M demonstrated complete inhibition of plaque formation on day 6. Bis(5-amidino-2-benzimidazolyl)methane at the same concentration reduced cell injury even when added to the system after 72 h of rickettsial infection. The reduction in morbidity in guinea pigs experimentally infected with *R. rickettsii* and treated with bis(5-amidino-2-benzimidazolyl)methane as compared with morbidity in infected, untreated animals, comprised delay in the onset of fever and slightly fewer febrile animals. Because bis(5-amidino-2-benzimidazolyl)methane had no effect on phospholipase A_2, the enzyme activity associated with penetration-induced cell injury, it is likely that a trypsin-like protease also plays an essential role either in the physiology of *R. rickettsii* or as its pathogenic mechanism.

Virions of several different genera require exposure to proteolytic enzymes to achieve full expression of their biological properties (7). As a corollary to this fact, protease inhibitors can be expected to have potential antiviral activity. This was substantiated by our recent discovery of the ability of synthetic low-molecular-weight inhibitors of trypsin-like proteases to block respiratory syncytial virus-induced cytopathology (2–4, 10). Although the site of action of these agents has not yet been determined, we presented strong evidence that their antiviral effect may be linked to their antiproteolytic properties. The most potent of the inhibitory agents, bis(5-amidino-2-benzimidazolyl)methane (BABIM), was shown to exert the following effects: (i) delay of penetration of virus into cells, (ii) blockage of virus-induced cell fusion, (iii) reduction of multiple-cycle yields of virus, and (iv) reduction in pathology and virus yield in experimentally infected animals.

Similar to viral diseases, rickettsial infection of cells involves a penetration step. This event is known to be enzyme mediated, but thus far only phospholipase A_2, and not a protease, has been shown to participate in the process (15, 23). Our experience with respiratory syncytial virus suggested application of the amidine inhibitors to the rickettsial system in search of evidence of proteolysis in the pathogenetic sequence. Such evidence was readily found in a study of the rickettsial plaque assay, and the results are the subject of this communication.

MATERIALS AND METHODS

Rickettsiae. Stocks of *R. rickettsii* (Sheila Smith strain) were cultivated by inoculation of the yolk sac of 5-day-old specific pathogen-free embryonated hen eggs (SPAFAS, Norwich, Conn.) with plaque-purified organisms provided by Charles L. Wisseman, Jr. (University of Maryland, Baltimore). Inoculated eggs were incubated at 35°C, and their yolk sacs were harvested 5 days after inoculation, 24 to 48 h after the death of the chick embryos. Yolk sacs, containing rickettsiae were homogenized in a Waring blender, diluted in sucrose phosphate glutamate (0.218 M sucrose, 0.0038 M KH_2PO_4, 0.0072 M K_2HPO_4, 0.0049 M glutamate, pH 7.0) (1) to a 1% suspension, and stored frozen at −70°C in 1-ml samples. Samples were titrated by plaque assay in chick embryo cell culture and found to contain 7×10^5 PFU/ml.

Plaque model. Flasks (Corning Glass Works, Corning, N.Y.) with 25-cm^2 monolayers of E6 clone Vero (African green monkey kidney) cells were inoculated with 0.1 ml of a suspension of *R. rickettsii* diluted 10^{-3} in brain heart infusion broth. The inoculum was adsorbed for 30 min before the addition of 5 ml of minimal essential medium (GIBCO Laboratories, Grand Island, N.Y.) containing 5% fetal bovine serum (Flow Laboratories, Inc., McLean, Va.), 0.02 M HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid) buffer, 2 mM L-glutamine, ca. 0.075% $NaHCO_3$ to final pH 7.3, 0.5% agarose (Sea Kem, FMC Corp., Marine Colloids Div., Rockland, Maine), and different concentrations of protease inhibitors. Flasks were incubated at 35°C for 4 days at which time 5 ml of an identical second overlay medium containing 0.01% neutral red was added. After further incubation, plaques were counted on various combinations of days 5, 6, and 7 (14, 16, 20–22).

Protease inhibitors. Inhibitors of trypsin-like proteases used in these experiments were BABIM, 1,2-bis(5-amidino-2-benzimidazolyl)ethane, 1,5-bis(5-amidino-2-benzimidazolyl)pentane, and 5-amidinoindole. They were synthesized as previously reported (5, 9).

Experimental design. Each of these aromatic amidines was incorporated into both the first and second overlay media of three flasks at concentrations of 10^{-4}, 10^{-5}, and 10^{-6} M, and plaque counts were compared with those of monolayers inoculated with the same rickettsial suspension and overlaid with medium containing no protease inhibitors. Control flasks included uninoculated, untreated monolayers and also uninfected monolayers treated with a 10^{-4} M concentration of the aromatic amidines for evaluation of toxicity. The mean plaque count was calculated for each concentration of

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TABLE 1. Effect of inhibitors of trypsin-like proteases on formation of plaques with *Rickettsia rickettsii*

Compound	Concn (M)	Effect of inhibitors on day[a]: 5 Plaques	5 Size	6 Plaques	6 Size	7 Plaques	7 Size	Trypsin K_i (μM)
Bis(5-amidino-2-benzimidazolyl)-methane	10^{-4}	—	—	0 ± 0	O	0 ± 0	O	0.017
	10^{-5}	—	—	0 ± 0	O	15.0 ± 3.5	S	
	10^{-6}	—	—	46.0 ± 1.5	S	52.0 ± 1.2	S	
	None	—	—	70.0 ± 5.0	N	68.5 ± 5.5	N	
1,2-Bis(5-amidino-2-benzinidazolyl)-ethane	10^{-4}	Toxic		Toxic		Toxic		4.68
	10^{-5}	21.7 ± 3.7	N	23.7 ± 3.8	N	23.0 ± 5.0	N	
	10^{-6}	22.3 ± 5.8	N	29.0 ± 7.6	N	31.7 ± 9.4	N	
	None	32.0 ± 3.0	N	37.3 ± 3.2	N	38.0 ± 2.6	N	
1,5-Bis(amidino-2-benzimidozolyl)-pentane	10^{-4}	—	—	0 ± 0	O	—	—	9.46
	10^{-5}	—	—	60.7 ± 5.2	S	—	—	
	10^{-6}	—	—	67.3 ± 2.4	N	—	—	
	None	—	—	71.0 ± 0.8	N	—	—	
5-Amidinoindole	10^{-4}	34.7 ± 2.0	N	51.7 ± 0.9	N	—	—	29.1
	10^{-5}	42.7 ± 1.2	N	56.7 ± 1.7	N	—	—	
	10^{-6}	Contam.		Contam.		—	—	
	None	39.5 ± 5.5	N	58.0 ± 6.0	N	—	—	

[a] Plaques are measured by mean number of plaques per flask ± standard error of the mean and by size: —, not examined; O, no plaques; S, small plaques; N, normal-size plaques; Contam., culture contaminated.

each amidine and was compared with the count of the *R. rickettsii*-infected flasks containing no amidine.

In a second experiment, the first overlay after inoculation of the monolayer with *R. rickettsii* contained no aromatic amidines. On day 3 (72 h after inoculation of rickettsiae), a second overlay was added to each flask. The second overlay contained BABIM at concentrations of 2×10^{-4}, 2×10^{-5}, and 2×10^{-6} M to achieve final protease inhibitor concentrations of 10^{-4}, 10^{-5}, and 10^{-6} M in the combined overlay medium.

Guinea pig experiment. Twenty-five adult (400- to 600-g) male guinea pigs (Hartley strain) were divided as follows. Ten animals were inoculated intraperitoneally with 330 50% tissue culture infective doses (ca. 38 50% guinea pig infectious doses) of *R. rickettsii* (Sheila Smith strain) and treated with BABIM at a dose of 15 mg/kg per day given daily by the intraperitoneal route beginning 30 min after rickettsial inoculation and continuing for 9 days; ten animals were inoculated intraperitoneally with 330 50% tissue culture infective doses of *R. rickettsii* and given no treatment, and five uninfected animals were given 15 mg of BABIM per kg daily via the intraperitoneal route for 9 days. Animals were examined daily, and rectal temperatures were measured with a battery-operated thermometer with a flexible probe (Telethermometer; Yellow Springs Instrument Co., Yellow Springs, Ohio).

Phospholipase A_2 assay. The procedure used was that described by Vigo et al. (12), in which hydrolysis of phospholipid-containing liposomes is followed spectrophotometrically at 340 nm. The assay mixtures of 1 ml of 0.1 M Tris-hydrochloride buffer (pH 7.2) contained 0.5 mg of dipalmitoyl-lecithin (as liposomes), 1 mM $CaCl_2$, and 10 U of phospholipase A_2 from *Naja naja* venom (Sigma Chemical Co., St. Louis, Mo.). The reaction was terminated by the addition of 1 ml of methanol containing 15 mM EDTA.

RESULTS

The effects of inhibitors of trypsin-like proteases on plaque count and size are presented in Table 1. There was a close correlation between the reduction in plaque count and the inhibition constants (K_i values) for trypsin (2). The most marked plaque reduction was observed with the most effective inhibitor of trypsin activity, BABIM, with no plaques being observed on day 6 after inoculation in the presence of 10^{-5} M of the amidine. Measurable plaque reduction was also observed at a concentration of 10^{-6} M BABIM, and the plaques present were smaller than untreated plaques. The least active inhibitor of trypsin, 5-amidinoindole, which has a K_i of 29.1 μM for trypsin (5), showed minimal effects on *R. rickettsii* plaque count and size. The monolayers treated with 1,5-bis(5-amidine-2-benzimidazolyl)pentane, having an intermediate K_i (9), showed evidence for reduction in cell injury caused by *R. rickettsii* as measured by plaque count and size. However, the effect was less than that of BABIM. At concentrations of 10^{-4} M and less, none of the aromatic amidines except 1,2-bis(5-amidino-2-benzimidazolyl)ethane caused cytotoxic effect on the Vero cells.

To determine whether delayed exposure to BABIM would still influence the cytopathic events in the monolayers, a second series of experiments was carried out. Here, the Vero cell culture was inoculated with *R. rickettsii*, and the establishment of infected foci was allowed to proceed normally by feeding with an initial overlay free of BABIM. On day 3 after inoculation, second overlays containing various concentrations of the inhibitor were added, and the resulting effects on the monolayer on day 6 and 7 were recorded (Table 2). As can be seen, there was complete suppression of plaque formation at a BABIM concentration of 10^{-4} M. At a concentration of 10^{-5} M, there was still a notable reduction in plaque count. However, the effect was less pronounced than it was when BABIM had been present in the medium immediately after the inoculation (Table 1).

BABIM was somewhat toxic for guinea pigs, causing a transient fever (≥40°C) early in the course of treatment (4 of 5 animals on day 3 and 2 of 5 animals on day 4) (Table 3). A similar transient fever was observed early in the course of BABIM treatment of animals infected with *R. rickettsii*. However, the temperatures of all guinea pigs returned to base line before day 5. Onset of fever was delayed; 5 of 10

TABLE 2. Effect of treating *Rickettsia rickettsii* plaque model with BABIM on day 3 after inoculation

BABIM concn (M)	Effect on day[a]: 6		7	
	Plaques	Size	Plaques	Size
10^{-4}	0 ± 0	O	0 ± 0	O
10^{-5}	31.3 ± 5.4	N	50.0 ± 13.0	S
10^{-6}	67.7 ± 9.0	N	82.7 ± 9.5	N
None	76.3 ± 3.8	N	82.0 ± 3.1	N

[a] Plaques are measured by mean number of plaques per flask ± standard error of the mean and by size: O, no plaques; N, normal plaque size; S, small plaques.

untreated animals had fever on day 5 as compared with no febrile animals in the BABIM-treated group. On day 6, 8 of 10 untreated animals were febrile as compared with only 2 of 10 BABIM-treated animals. Four febrile animals in each group died during the course of the rickettsial disease.

BABIM was found to have no effect on the hydrolysis of dipalmitoyl-lecithin liposomes by phospholipase A_2 from *Naja naja* venom.

DISCUSSION

The order of effectiveness of aromatic amidines in reducing plaque formation by *R. rickettsii* follows their order of effectiveness in inhibiting trypsin and several other trypsin-like proteases and corresponds to their order of effectiveness in blocking cell fusion by respiratory syncytial virus in vivo (2, 5, 10). This parallelism of activities at low concentrations argues strongly for the antirickettsial effect resulting from the action of compounds as protease inhibitors on a trypsin-like enzyme. However, it was also necessary to consider the possibility that amidines might act by inhibition of phospholipase A_2, especially since a cell penetration phospholipase appears to be an important rickettsial pathogenic mechanism and a protease inhibitor of a different structure had previously been shown to suppress phospholipase A_2 activity (8). We showed this possibility to be unlikely by demonstrating that BABIM at a concentration of 10^{-4} M has no effect on phospholipase A_2 activity (from *Naja naja* venom). Of course, the linkage of a trypsin-like protease in a chain of enzymes that includes phospholipase A_2 cannot be excluded (11). In fact, trypsin activation is necessary for all mammalian pancreatic phospholipases A_2 (13). Some of the other possible mechanisms by which a trypsin-like protease might fit into the scheme of rickettsial injury to cells, besides proteolytic activation of the penetration mechanism-associated phospholipase, include direct proteolytic attack on the host cell membrane either during entrance into the cell or on release from the cell or an essential intracellular catabolic function.

The reduction of plaque counts by the addition of BABIM 72 h after the establishment of rickettsia-infected foci documents that the protease inhibitors are not merely preventing initial rickettsial infection and suggests that protease inhibitors may be blocking a rickettsial function essential to expression of the pathogenic mechanism. The hypothesis that a protease-associated pathogenic mechanism is blocked by BABIM is also supported by the delay of plaque formation by a 10^{-5} M concentration of BABIM with delayed appearance of several small plaques on day 7. Rickettsiae survived the BABIM treatment and caused formation of a few plaques, presumably after protease activity overcame the protease inhibitory activity. Because fatal cases of Rocky Mountain spotted fever are often diagnosed and treated too late in the course of disease for rickettsiostatic antimicrobial agents to prevent the demise of the patients (6, 17–19), additional inhibition of rickettsial injury to the host would benefit such critically ill patients. Consideration of the possible use of inhibitors of trypsin-like proteases for the treatment of rickettsial diseases would require investigation of these drugs in other animal models, further study of the mechanism of action of protease inhibitors on the rickettsia-host cell interaction, and information on the toxicity of these compounds in humans, especially on the inflammatory and coagulation mechanisms: complement, kallikrein, coagulation, and fibrinolysis.

Data on the toxicity of BABIM include the acute 50% lethal dose for cotton rats of 136 mg of BABIM per kg and a dosage for cotton rats of 30 mg of BABIM per kg daily for 7 days without ill effects. Current pharmacological studies of

TABLE 3. Effect of BABIM on experimental infection of guinea pigs with *Rickettsia rickettsii*

Day	No.[a] of: Rickettsia-infected guinea pigs: BABIM treated			Rickettsia-infected guinea pigs: No treatment			Uninfected guinea pigs: BABIM treated		
	Afebrile	Febrile	Dead	Afebrile	Febrile	Dead	Afebrile	Febrile	Dead
1	10	0	0	10	0	0	5	0	0
2	6	4 (40.1)	0	10	0	0	5	0	0
3	2	8 (40.2)	0	10	0	0	1	4 (40.1)	0
4	6	4 (40.1)	0	10	0	0	3	2 (40.1)	0
5	10	0	0	5	5 (40.6)	0	5	0	0
6	8	2 (40.8)	0	2	8 (40.7)	0	5	0	0
7	6	4 (40.7)	0	2	8 (40.7)	0	5	0	0
8	5	4 (40.7)	1	2	8 (40.7)	0	5	0	0
9	4	5 (40.7)	1	3	7 (40.8)	0	5	0	0
10	6	3 (40.7)	1	2	7 (40.8)	1	5	0	0
11	4	3 (40.5)	3	4	5 (40.7)	1	5	0	0
12	6	1 (40.8)	3	5	3 (40.6)	2	5	0	0
13	6	1 (40.4)	3	4	3 (40.4)	3	5	0	0
14	6	1 (40.4)	3	4	3 (40.3)	3	5	0	0
Final	6	0	4	6	0	4	5	0	0

[a] The values in parentheses are the mean temperatures of febrile guinea pigs.

rats that received a dose of 20 mg of BABIM per kg daily and of mice that received a dose of 20 mg of BABIM per kg daily have not detected any toxicity. These observations of protease inhibitors blocking rickettsial injury to cells offer interesting new hypotheses for studying rickettsial pathogenesis. At this point, they should be used as tools for elucidating rickettsial physiology and pathogenic mechanisms.

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